Remarks/Argument

This response is being filed concurrently with a Request for Continued Examination. Per the petition and fee submitted herewith, the Applicants hereby extend the time period for responding to the outstanding Office Action by three months to February 11, 2005. Please charge any further fee which may be due, or credit any overpayment, to deposit account no.50-2719.

Claims 1-31 and 33 have been previously canceled. Claim 32 is pending in the application and has been amended. Support for amended claim 32 is found on page 7, first paragraph of the specification and in Fig. 8. No new matter has been added by this amendment.

Priority

The current status of the earlier-filed applications from which this application claims priority has been inserted into the first sentence of the specification by amendment. The Applicants believe that all conditions for receiving the benefit of an earlier filing date under 35 U.S.C. 120 have been satisfied.

Sequence Compliance

The sequence identifiers for the sequences referred to in the figure descriptions of Figures 5-7, 8A-8B, 10[1] and 11A-11D have been inserted by amendment. A new sequence listing was not submitted, as the sequence listing on file contains all sequences referred to in the specification and is believed to comply with the sequence rules.

Response to the section 112, 1st paragraph enablement rejection

Claim 32 has been rejected under 35 U.S.C. 112, 1st paragraph as allegedly being non-enabled because the claim does not recite a specific phenotype for the transgenic TASK knockout mouse. According to pg. 4 of the Office Action, "one would not know when they had

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[1] The Office Action refers to Figures "8A-8F" and "10A-10B." However, this application contains only Figs. 8A-8B, and Fig. 10.

attained the claimed mouse or how to use it, because it is not known what phenotype the claimed mouse should exhibit." Applicants respectfully disagree.

A claim is enabled when the specification teaches one skilled in the art how to make and use the claimed invention without undue experimentation. Here, claim 32 is directed to a transgenic knock-out mouse which is deficient in the expression of a potassium transport channel which comprises SEQ ID NO: 5. There is no recitation in the claim that the transgenic knock-out mouse must have a certain phenotype, other than it be deficient in the expression of TASK.

The Office Action states that the phenotype of the claimed TASK knockout mouse would be unpredictable, that determining the phenotype would require undue experimentation, and that one skilled in the art would not know how to use the claimed transgenic mouse. However, the concerns expressed in the Office Action relating to the possible phenotypic characteristics of the claimed mouse are misplaced, because the claims do not include or require these limitations. The appellants need only provide an enabling disclosure for the claimed invention. In re Vaeck, 20 USPQ2d 1438, 1445 (Fed. Cir. 1991); In re Fisher, 166 USPQ 18, 24(CCPA 1970). There is no requirement for a disclosure supporting possible, but unclaimed, phenotypic characteristics of the claimed mouse. A virtually identical situation presented itself in the unpublished Board of Patent Appeals and Interferences opinion of Ex parte Chen, 61 USPQ2d 1025 (BPAI 2001); copy enclosed. In Chen, claims to transgenic carp which contained no phenotype limitations were considered enabled, despite the Examiner's insistence that phenotype was unpredictable and that phenotypic characteristics be read into the claims. This decision is NOT being cited as precedent, but is rather brought to the Examiner's attention to illustrate that the issues under discussion here are well-developed and have already been considered by the Board.It is therefore not necessary for one skilled in the art to identify the phenotype of the claimed mouse, beyond determining whether the mouse is deficient in TASK expression.

Techniques for determining whether transgenic mice are deficient in TASK expression are well-known and routine in the art. For example, the amount of TASK mRNA present in tissues of the transgenic mouse can be determined using standard molecular biology techniques such as Northern blot, *in situ* hybridization or reverse-transcriptase PCR ("RT-PCR"). The amount of TASK protein present in tissues of the transgenic mouse can be determined using standard immunological techniques such as Western blot or ELISA. See, *e.g.*, the Results

section on pg. 2684, col. 2 of Herteux et al., 2004, *EMBO J*, 23: 2684-2695 (copy enclosed), which discusses the use of *in situ* hybridization, RT-PCR and immunological techniques to confirm the lack of expression of TREK-1 mRNA and protein in transgenic TREK-1 knockout mice. One skilled in the art would have been aware, at the time this application was filed, of how to employ standard molecular biology or immunological techniques to identify transgenic mice which are deficient for TASK expression that fall within the scope of claim 32. The use of Northern blot, *in situ* hybridization, RT-PCR or immunological techniques (such as are discussed in Herteux et al.) to determine gene expression in transgenic mice were routine at the time this application was filed, and would not have been considered undue experimentation.

Moreover, a transgenic mouse deficient for TASK expression falling within the scope of claim 32 has actually been produced. See Linden et al., 2004, *Program No. 966.1. 2004 Abstract Viewer/Itinerary Planner*, Washington, DC: Society for Neuroscience Online (abstract only; copy enclosed). These TASK knockout mice were used to characterize the sensitivity of TASK potassium channels to certain anesthetics and potassium channel agonists. The specification discloses on pg. 28, lines 12-18 that the claimed TASK knockout mice can be used to prepare live models for studying conditions associated with TASK channels. Thus, the actual use to which transgenic mice falling within the scope of claim 32 were put is a use which was expressly disclosed in the specification.

Transgenic TASK knockout mice as recited in claim 32 can therefore be made and used by following the teachings of the specification, without undue experimentation. As claim 32 does not recite any phenotypic characteristics for the transgenic mice other than a deficiency in TASK expression, the alleged unpredictability of the transgenic mouse phenotype discussed in the Office Action is not relevant. Claim 32 is therefore enabled by the specification, and the Applicants respectfully request withdrawal of the 35 U.S.C. 112, 1st paragraph rejection.

Response to Section 112, 2nd paragraph rejection

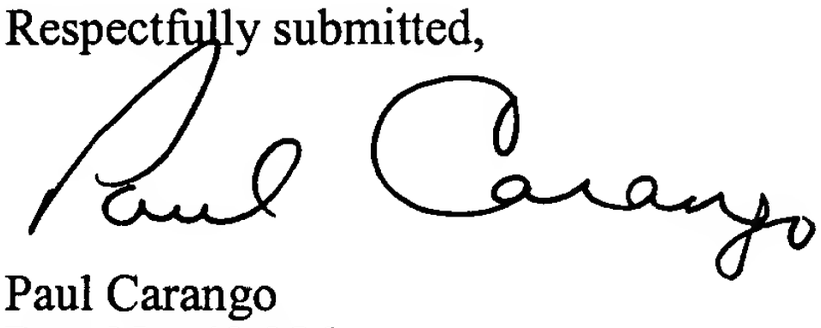
Claim 32 has been rejected under 35 U.S.C. 112, 2nd paragraph as being indefinite for reciting a potassium transport channel encoded by a nucleic acid sequence comprising SEQ ID NO: 5. As pointed out by the Examiner, SEQ ID NO: 5 is an amino acid sequence. Claim 32 has been amended to specify that the claimed potassium transport channel comprises SEQ ID

NO: 5. Applicants respectfully request that the section 112, 2nd paragraph rejection of claim 32 be withdrawn.

Conclusion

In view of the foregoing, the Applicants respectfully submit that the application is now in a condition for allowance, which is respectfully requested.

Respectfully submitted,



Paul Carango
Reg. No. 42,386

PC:rb
(215) 656-3320

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TREK-1, a K^+ channel involved in neuroprotection and general anesthesia

C Heurteaux[1], N Guy[1], C Laigle, N Blondeau, F Duprat, M Mazzuca, L Lang-Lazdunski, C Widmann, M Zanzouri, G Romey and M Lazdunski*

Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, Institut Paul Hamel, Sophia-Antipolis, Valbonne, France

TREK-1 is a two-pore-domain background potassium channel expressed throughout the central nervous system. It is opened by polyunsaturated fatty acids and lysophospholipids. It is inhibited by neurotransmitters that produce an increase in intracellular cAMP and by those that activate the Gq protein pathway. TREK-1 is also activated by volatile anesthetics and has been suggested to be an important target in the action of these drugs. Using mice with a disrupted TREK-1 gene, we now show that TREK-1 has an important role in neuroprotection against epilepsy and brain and spinal chord ischemia. Trek1$^{-/-}$ mice display an increased sensitivity to ischemia and epilepsy. Neuroprotection by polyunsaturated fatty acids, which is impressive in Trek1$^{+/+}$ mice, disappears in Trek1$^{-/-}$ mice indicating a central role of TREK-1 in this process. Trek1$^{-/-}$ mice are also resistant to anesthesia by volatile anesthetics. TREK-1 emerges as a potential innovative target for developing new therapeutic agents for neurology and anesthesiology.

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Subject Categories: neuroscience; molecular biology of disease
Keywords: epilepsy; ischemia; neuroprotection; 2P domain K^+ channel; volatile anesthetics

Introduction

Two-pore-domain potassium channels (K_{2P} channels) form a novel class of K^+ channels identified in various types of neurons (Kim *et al*, 1995; Wei *et al*, 1996; Lesage and Lazdunski, 2000; Talley *et al*, 2003). They are open at membrane potentials across the physiological range and are therefore likely to contribute to the background or leak currents that help set the resting membrane potential and oppose depolarizing influences. They are key components in shaping the characteristics of neuronal excitability. TREK-1 (Fink *et al*, 1996) is expressed throughout the central nervous system (Fink *et al*, 1996; Lauritzen *et al*, 2000; Maingret *et al*, 2000b; Hervieu *et al*, 2001; Talley *et al*, 2001) and is an important member of this family. It is the probable mammalian homolog of the Aplysia S-type K^+ channel (Siegelbaum *et al*, 1982; Patel *et al*, 1998), a channel involved in simple forms of learning and memory. TREK-1 is activated by membrane stretch and intracellular acidification (Patel *et al*, 1998; Maingret *et al*, 1999b). TREK-1 is opened by arachidonic acid and other polyunsaturated fatty acids (PUFAs) as well as lysophospholipids (LPLs) (Patel *et al*, 1998; Maingret *et al*, 2000b). On the other hand, PUFAs and LPLs are potent protective agents against forebrain ischemia and seizures, and it has been proposed that this effect results, at least in part, from their action on TREK channels (Lauritzen *et al*, 2000; Blondeau *et al*, 2001, 2002). TREK-1 probably has a central role in the control of excitability by a variety of neurotransmitters. TREK-1 is potently inhibited by neurotransmitters that produce an increase in intracellular cAMP (Patel *et al*, 1998) and also by those that activate the Gq protein pathway (Lesage *et al*, 2000; Chemin *et al*, 2003). The inhibition of TREK channels by glutamate via the activation of group I Gq-coupled metabotropic glutamate receptors requires PTX-insensitive G proteins coupled to phospholipase C (Chemin *et al*, 2003). TREK-1 is also activated by volatile anesthetics and suggested to be a target in the action of these drugs (Patel *et al*, 1999). This paper definitively shows that TREK-1 plays a major role in the PUFAs/LPLs-induced neuroprotection against epilepsy and ischemia and that TREK-1-deficient mice display resistance to anesthesia.

Results

Generation and characterization of TREK-1 null mice

The TREK-1 gene of mice was disrupted through homologous recombination using a Cre/loxp-based strategy (Figure 1A). The CRE-mediated excision of exon 3 led to the deletion of the first transmembrane domain of the TREK-1 channel. Heterozygous matings produced offspring with normal Mendelian ratios (Figure 1B and C). Homozygous (Trek1$^{-/-}$) mutant mice were healthy, fertile and did not display any visible morphological differences. PCR amplification of testicular cDNA (a tissue where TREK-1 is abundant; Hervieu *et al*, 2001; Talley *et al*, 2001) showed that the null mutant only expressed a truncated transcript (Figure 1D). Sequencing of this transcript confirmed that it results from the deletion of the 311 nucleotides of the targeted exon (Figure 1D). The brain morphology of Trek1$^{-/-}$ mice appeared normal. In brain regions known to express the KCNK2 gene, no TREK-1 messenger RNA was detected by *in situ* hybridization using a probe recognizing the 3′-end of the mRNA (Figure 1E). The absence of the TREK-1 protein in null mutants was confirmed by the lack of immunoreactivity to specific anti-TREK-1 antibody (Maingret *et al*, 2000a) in brain areas such as the cortex or the hippocampus where it is highly expressed (Figure 1F).

*Corresponding author. Institut de Pharmacologie Moléculaire et Cellulaire, CNRS-UMR 6097, Institut Paul Hamel, 660 Route des Lucioles, Sophia-Antipolis, 06560 Valbonne, France.
Tel.: +33 493 957702/03; Fax: +33 493 957704;
E-mail: ipmc@ipmc.cnrs.fr
[1]These authors contributed equally to this work

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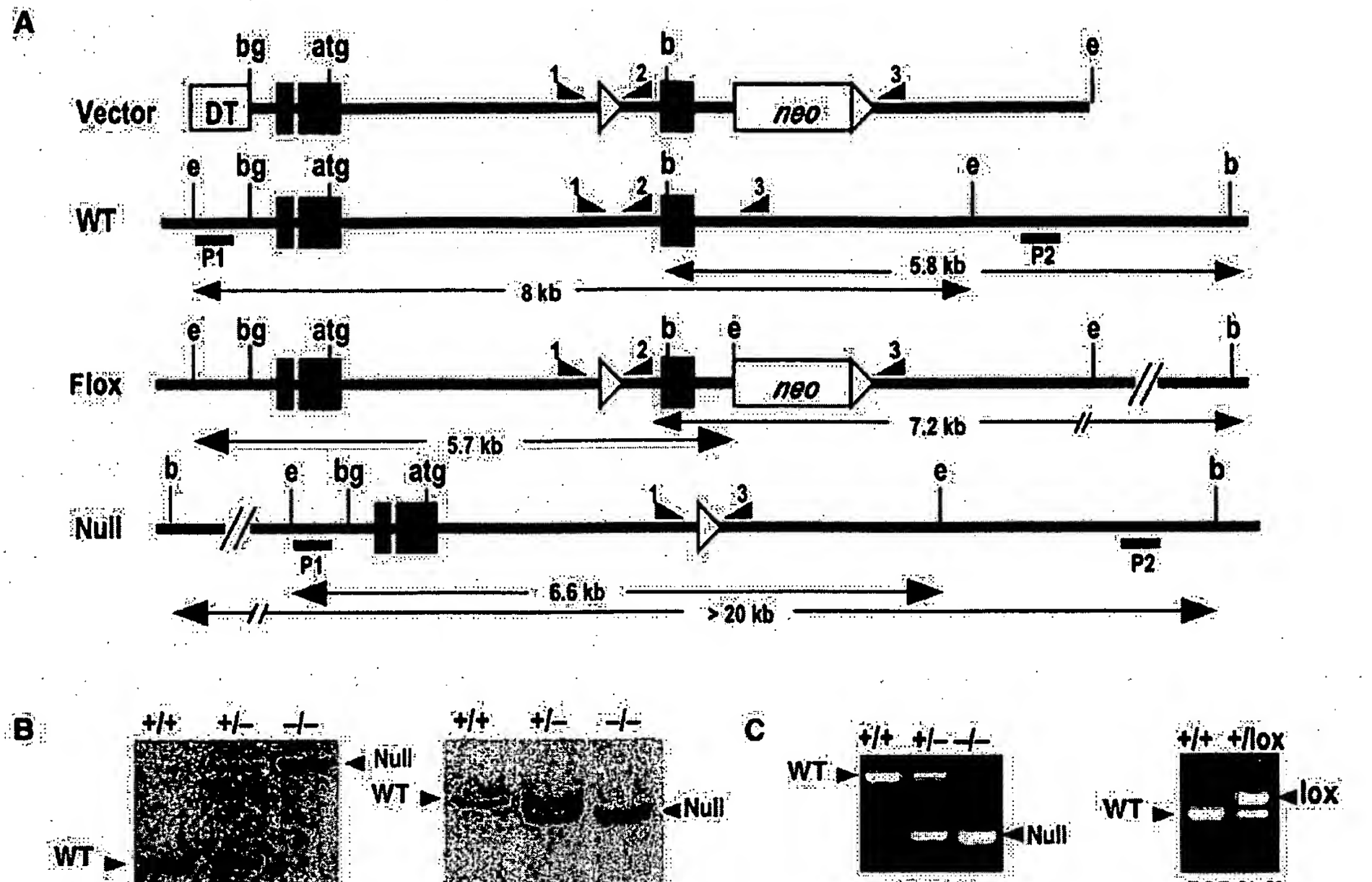

Figure 1 Disruption of the KCNK2 gene. (**A**) Targeting vector (►=loxp), native (WT) and recombined floxed (Flox) alleles. External probes used to characterize homologous recombination are designated as P1 and P2. Arrowheads (1–3) display locations of the primers used for PCR analysis of the different products. Double-headed arrows indicate the expected size of restriction fragments for Southern analysis (bg = *Bgl*II; b = *Bam*HI; e = *Eco*RI). (**B**) Southern blot analysis of *Eco*RI- and *Bam*HI-digested tail DNA from wild-type (+/+), heterozygous (+/−) or homozygous (−/−) KCNK2 mice probed with P1 and P2, respectively. (**C**) PCR amplification from tail genomic DNA. (**D**) PCR amplification from +/+, +/− and −/− mouse testis cDNA with primers surrounding the deletion. (**E**) *In situ* hybridization analysis shows the lack of mRNA expression in $Trek^{-/-}$ mouse brain on X-ray films. (**F**) Immunocytochemical TREK-1 staining in neocortex (Cx) and hippocampal CA3 subfield sections using a specific α-TREK-1 antibody (Lauritzen *et al*, 2000).

The TREK-1 mutation did not interfere with the mRNA expression in brain and cerebellum of other K_{2P} channels and of the GABAα6 subunit whose deletion causes an increased expression of TASK-1, another K_{2P} channel (Brickley *et al*, 2001) (Figure 2A). There was no compensatory upregulation of genes for other neuronal K_{2P} channels such as TWIK-1, TREK-2, TRAAK, TASK-1, TASK-3 or the GABAα6 subunit in Trek1$^{-/-}$ mice ($P<0.01$).

Primary behavioral testings (see Supplementary Materials and methods) showed that the TREK-1-deficient mice did not display any abnormal phenotype in appearance (Figure 2B). There was no difference in skin color, body tone or body weight. Trek1$^{-/-}$ mice did not display any abnormalities in body position, respiration or spontaneous activity. Stereotypies or tremor were not observed. There was no difference in frequency and volume of defecation or urination. Locomotor activity of the Trek1$^{-/-}$ mutant was not different from Trek1$^{+/+}$ control in the open field test as well as in the rotarod. No difference was seen in the touch escape response or in the positional passivity test. Recordings of reflexes and autonomic functions did not show any significant differences. Scorings were comparable in the visual placing test, grip strength, corneal and pinna reflex and in the righting reflex. No significant difference was seen between Trek1$^{+/+}$ and Trek1$^{-/-}$ mice in the object recognition test.

For comparative purpose, we have also deleted the TRAAK gene (see Supplementary Materials and methods and Supplementary Figure 1) to be able to evaluate the respective properties of Trek1$^{-/-}$ and Traak$^{-/-}$ mice. The TRAAK channel is closely related to the TREK-1 channel. Like TREK-1, it is a background outward rectifier K^+ channel, opened by membrane stretch, cell swelling and activated by PUFAs and LPLs. However, unlike TREK-1, the TRAAK channel is not activated by intracellular acidification (Maingret *et al*, 1999b) nor volatile anesthetics (Patel *et al*, 1999) and not inhibited by neurotransmitters that increase cAMP via a protein kinase A-dependent phosphorylation process (Fink

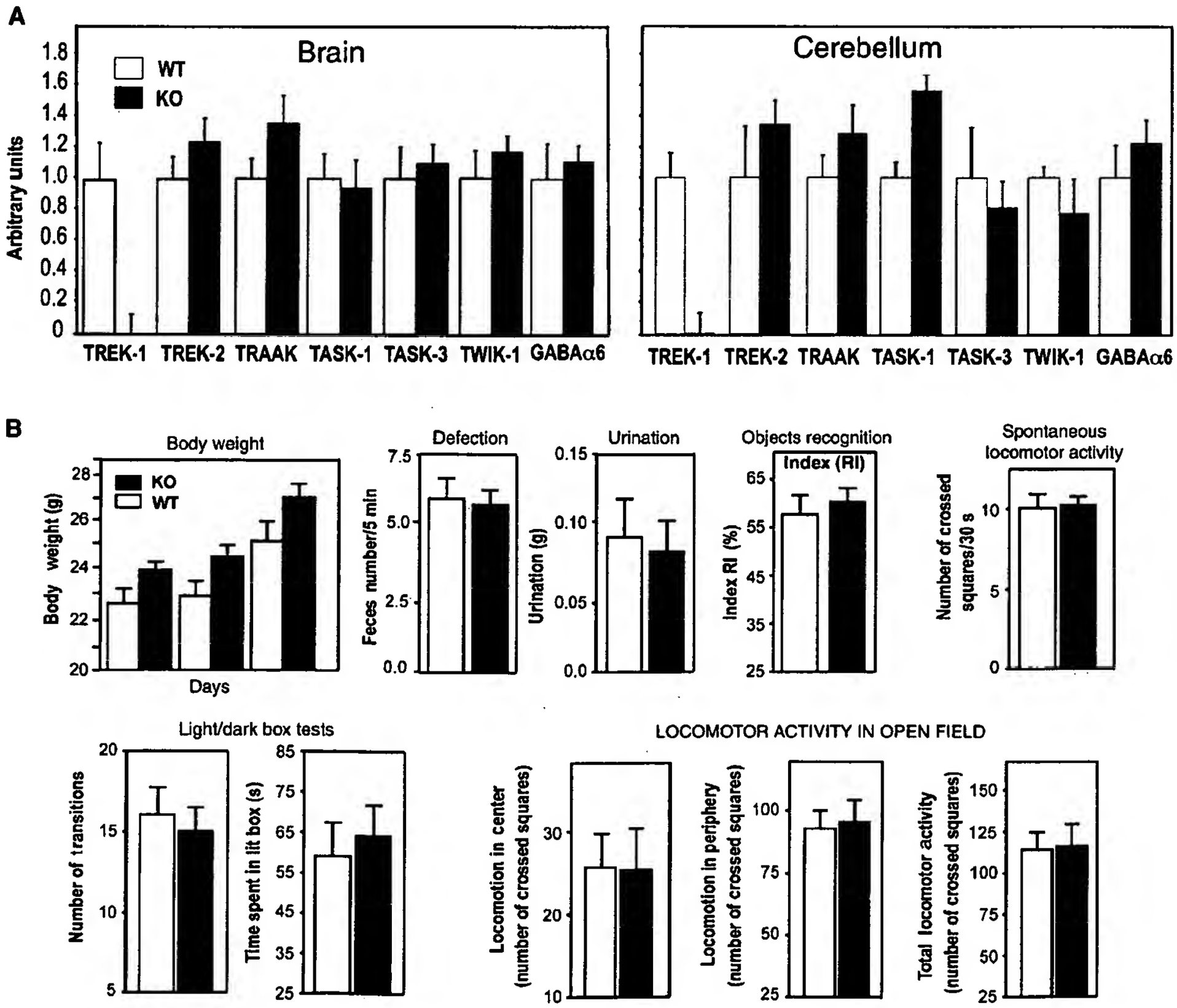

Figure 2 Characterization of TREK-1 null mice. (**A**) Relative expression of TREK-1, TREK-2, TRAAK, TASK-1, TASK-3 and GABAα6 mRNA levels in brain and cerebellum from Trek1$^{-/-}$ and Trek1$^{+/+}$ mice. Mean levels of gene expression, normalized to cyclophilin D, are displayed in arbitrary units on the vertical axis ($n=3$ mice, $P<0.01$, Student's t-test). (**B**) Primary behavioral test battery showing the lack of abnormal phenotype in TREK-1-deficient mice. Results are expressed as mean±s.e.m. Statistical significance was set at $P<0.05$ (Student's t-test or a Mann–Whitney test).

et al, 1998; Maingret *et al*, 1999a) or by those that activate the Gq protein pathway (Chemin *et al*, 2003).

Electrophysiological recordings

To test whether TREK-1 currents could be recorded in neurons from wild-type mice and were absent in neurons from TREK-1 null mice, we performed patch clamp recordings in striatal neurons in culture. These neurons were chosen because they strongly express TREK-1 but not TREK-2 or TRAAK channels (Hervieu *et al*, 2001; Talley *et al*, 2001), two K_{2P} channels that are also activated by membrane stretch, PUFAs and LPLs (Lesage and Lazdunski, 2000; Lesage *et al*, 2000; Patel and Honoré, 2001). In the striatum, the primary type accounting for 85% of the neurons is the GABAergic medium-size spiny neuron (Kita and Kitai, 1988). Using an antibody against GABA, we have checked that most neurons in our culture were indeed GABAergic (data not shown). The resting membrane potential of the striatal neurons from Trek1$^{+/+}$ and Trek1$^{-/-}$ mice was not significantly different (Student's *t*-test, $P=0.0586$) with -47.2 ± 1.6 mV ($n=30$) and -51.5 ± 7.5 mV ($n=26$), respectively. Neurons with resting membrane potential less negative than -30 mV were discarded. Using the inside-out configuration and in the presence of K^+ channels blockers (TEA, 4-AP and glibenclamide), a native TREK-1-like current was regularly recorded in cultures from wild-type mice. This current was reversibly activated by 10 μM arachidonate (AA) (Figure 3A) and by internal acidification (Figure 3B), as previously described (Maingret *et al*, 1999b, 2000b). The conductance was 55.8 ± 0.9 pS at $+50$ mV ($n=6$), which is close to the conductance of the cloned TREK-1 (Patel *et al*, 1998). The outwardly rectifying current reversed around the potassium equilibrium potential (Figure 3C). Like TREK-1 (Patel *et al*, 1998), the native current was also activated by membrane stretch (Figure 3D). The effect of volatile anesthetics was also studied on the TREK-like current recorded in striatal cultures from wild-type mice (Figure 3E, inset) and in TREK-1-transfected COS cells (Supplementary Figure 2A and B). Halothane in striatal neurons (Figure 3E, inset) as well as halothane and sevoflurane in COS cells (Supplementary Figure 2A and B) highly stimulated a TREK-1 channel activity. The loss of functional TREK-1 channels in TREK-1 null mutants was demonstrated by outside-out patch clamp recordings in striatal neurons. Figure 3E and F shows that in the presence of TEA and 4-AP to block voltage-dependent K^+ channels, there was no expression of basal current in wild-type neurons and in null mutants. Upon perfusion with the TREK-1 activator AA (20 μM), a robust TREK-1-like current was recorded in Trek1$^{+/+}$ neurons, whereas no significant variation was observed in Trek1$^{-/-}$ neurons. This electrophysiological analysis confirmed (i) that the TREK-1 deletion had taken place and (ii) that there was no compensatory upregulation of genes for other neuronal K_{2P} channels.

Role for the TREK-1 channel in the control of epileptogenesis

The high level of TREK-1 channel expression in the cortex and thalamic nuclei and its colocalization on GABAergic cortical and hippocampal interneurons, which are inhibitory to pyramidal cell activity (Hervieu *et al*, 2001; Talley *et al*, 2001), suggest a possible involvement of the TREK-1 channel in the control of epileptic seizures. To analyze the seizure

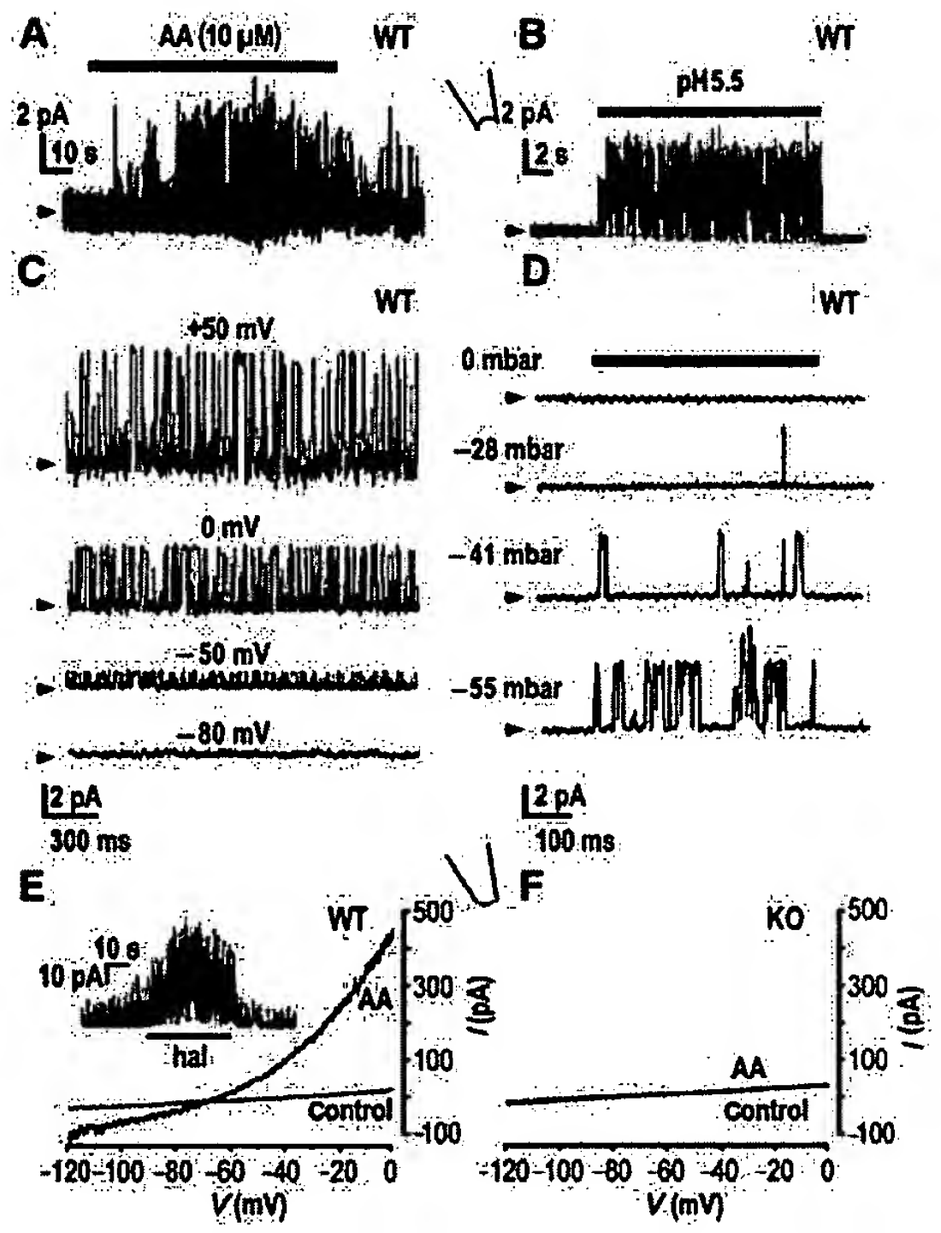

Figure 3 Patch clamp recordings in striatal neurons from Trek1$^{+/+}$ and Trek1$^{-/-}$ mice. (**A**) Activation of the TREK-like current by 10 μM AA. (**B**) Activation of the TREK-like current by internal acidification to pH 5.5. Currents in (A, B) were recorded in inside-out configuration at 0 mV. (**C**) Single channel currents recorded as in (A) at various potentials as indicated. (**D**) Activation by membrane stretch recorded as in (A) at various negative pressures as indicated. (**E**) Typical TREK-like current recorded in outside-out configuration before (control) and after activation by 10 μM AA in striatal neurons from wild-type mice (WT). Values are average of two consecutive current traces elicited with voltage ramps starting from 0 mV down to -120 mV, from a holding potential of 0 mV. Inset: Effect of 2 mM halothane (hal) on TREK-like activity recorded at 0 mV in outside-out configuration. (**F**) Same recordings in neurons from TREK-1 knockout mice (KO).

susceptibility of Trek-1-deficient mice, we used the response to kainic acid (KA, an agonist of glutamate receptor) and to pentylenetetrazol (PTZ, a $GABA_A$ receptor antagonist), as an overall index of neuronal network excitability. Trek1$^{+/+}$ and Trek1$^{-/-}$ mice were injected intraperitoneally with epileptogenic doses of KA (22 mg/kg) or PTZ (40–55 mg/kg) and the degree of seizures was scored (Tsirka *et al*, 1995). Trek1$^{-/-}$ mice were much more vulnerable to KA-induced seizures than Trek1$^{+/+}$ mice as assessed by either seizure score or mortality rate (Figure 4A). More than 75% of the mutant mice died within 3 days of KA administration, compared with 3% of Trek1$^{+/+}$ mice, and the average maximum intensity of seizures observed in Trek1$^{-/-}$ mice increased by 33%. A comparison of electroencephalogram (EEG) patterns in the hippocampus of Trek1$^{+/+}$ and Trek1$^{-/-}$ mice is shown in Figure 4F. A spectral analysis of EEG activity shows that 45 min following KA treatment (22 mg/kg), Trek1$^{-/-}$ mice developed generalized convulsive seizures with the appearance of bilateral spike-wave discharges with spike frequencies and amplitudes higher than in Trek1$^{+/+}$ mice (Figure 5A and B).

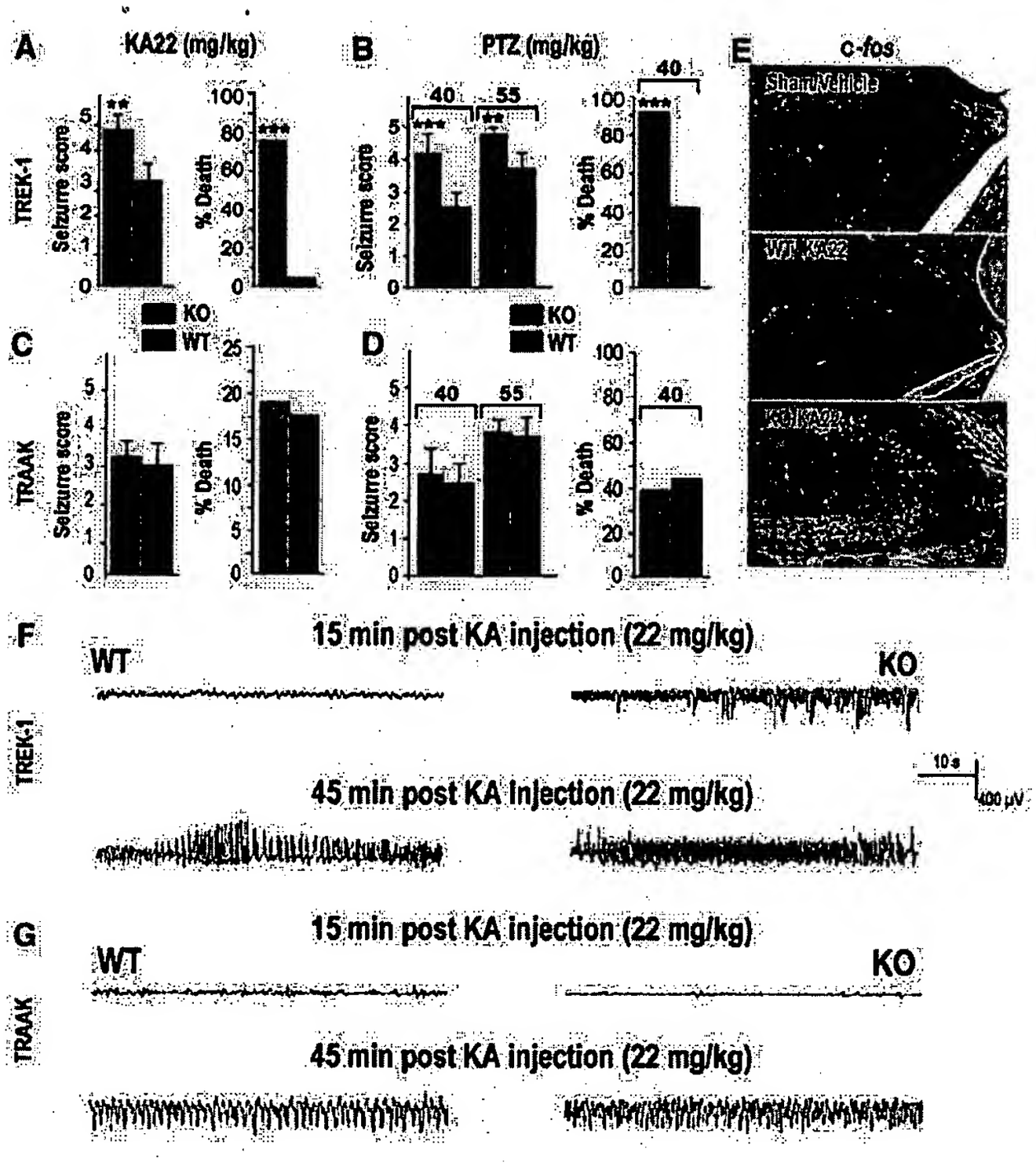

Figure 4 Increased susceptibility to epileptic agents in TREK-1-deficient mice. (**A, B**) Seizure behavior and mortality rate in wild-type and mutant TREK-1 mice after KA (A) or PTZ injection (B). (**C, D**) Seizure behavior and mortality rate in wild-type and mutant TRAAK mice after KA (C) or PTZ (D) injection. Seizures were scored for 2 h after intraperitoneal injection with KA (22–28 mg/kg) or PTZ (40–55 mg/kg). Seizures were ranked as follows: 1, immobility; 2, myoclonic jerks of the neck and head with brief twitching movements; 3, unilateral clonic activity; 4, bilateral forelimb tonic and clonic activity; 5, generalized tonic–clonic activity with loss of postural tone including death from continuous convulsions. Values represent mean ± s.e.m. of the maximum seizure intensity recorded for each mouse ($n = 20$ per genotype). *Significantly different from vehicle-treated wild type (KA treatment 22 mg/kg), $^{**}P < 0.001$, $^{***}P < 0.0001$, ANOVA followed by Tukey's multiple comparison test. (**E**) Increased expression of c-*fos* protein in CA3 pyramidal neurons in Trek1$^{-/-}$ mice 120 min after KA treatment (22 mg/kg). (**F**) EEG following KA (22 mg/kg) showing the increased KA susceptibility of Trek1$^{-/-}$ mice as compared to Traak$^{-/-}$ mice (**G**) ($n = 10$ per genotype).

Trek1$^{-/-}$ mice also showed an increased sensitivity to PTZ-induced seizures (Figure 4B). Unlike the slow progression of motor symptoms observed in the KA-induced seizures, PTZ induced abrupt general tonic–clonic seizures within 5 min of injection. At a dose of 55 mg/kg, more than 90% of Trek1$^{-/-}$ mice died from continuous tonic–clonic convulsions, whereas 60% of Trek1$^{+/+}$ mice survived (Figure 4B).

Activation of c-*fos*, in regions susceptible to kainate injection, is routinely used as a biochemical marker of neuronal excitability (Smeyne *et al*, 1992). The expression of the c-*fos* protein was drastically enhanced in Trek1$^{-/-}$ mice compared to Trek1$^{+/+}$ mice, particularly in CA3 subfield at 120 min after KA injection (Figure 4E).

A comparative study was carried out with the TRAAK channel. TRAAK-deficient mice did not display an increased sensitivity to epilepsy (Figures 4C, D and G and 5C). Taken together, all these results show that, unlike TRAAK null mice, TREK-1-deficient mice are hypersensitive to kainate and PTZ-induced seizures and point to TREK-1 as a key target for epileptogenesis.

TREK-1 channel in brain and spinal chord ischemia and its major role in the neuroprotection provided by PUFAs and LPLs

Linolenic acid (LIN) or lysophosphatidylcholine (LPC) at a dose of 500 nmol/kg injected 30 min before the KA administration induced a potent decrease of the seizure activity in Trek1$^{+/+}$ mice but had no effect in Trek1$^{-/-}$ mice (Figure 6A and B). The seizure score or the mortality rate shows that LIN- or LPC-injected Trek1$^{+/+}$ mice were much less vulnerable to KA-induced seizures than vehicle-injected Trek1$^{+/+}$ mice, while LIN- or LPC-injected Trek1$^{-/-}$ mice were not protected (Figure 6A). More than 78% of the mutant mice treated with LIN or LPC died within 3 days of KA22 administration, compared with 3% of LIN- or LPC-injected Trek1$^{+/+}$ mice, and the average maximum intensity of seizures observed in treated Trek1$^{-/-}$ mice increased by 38%. EEG

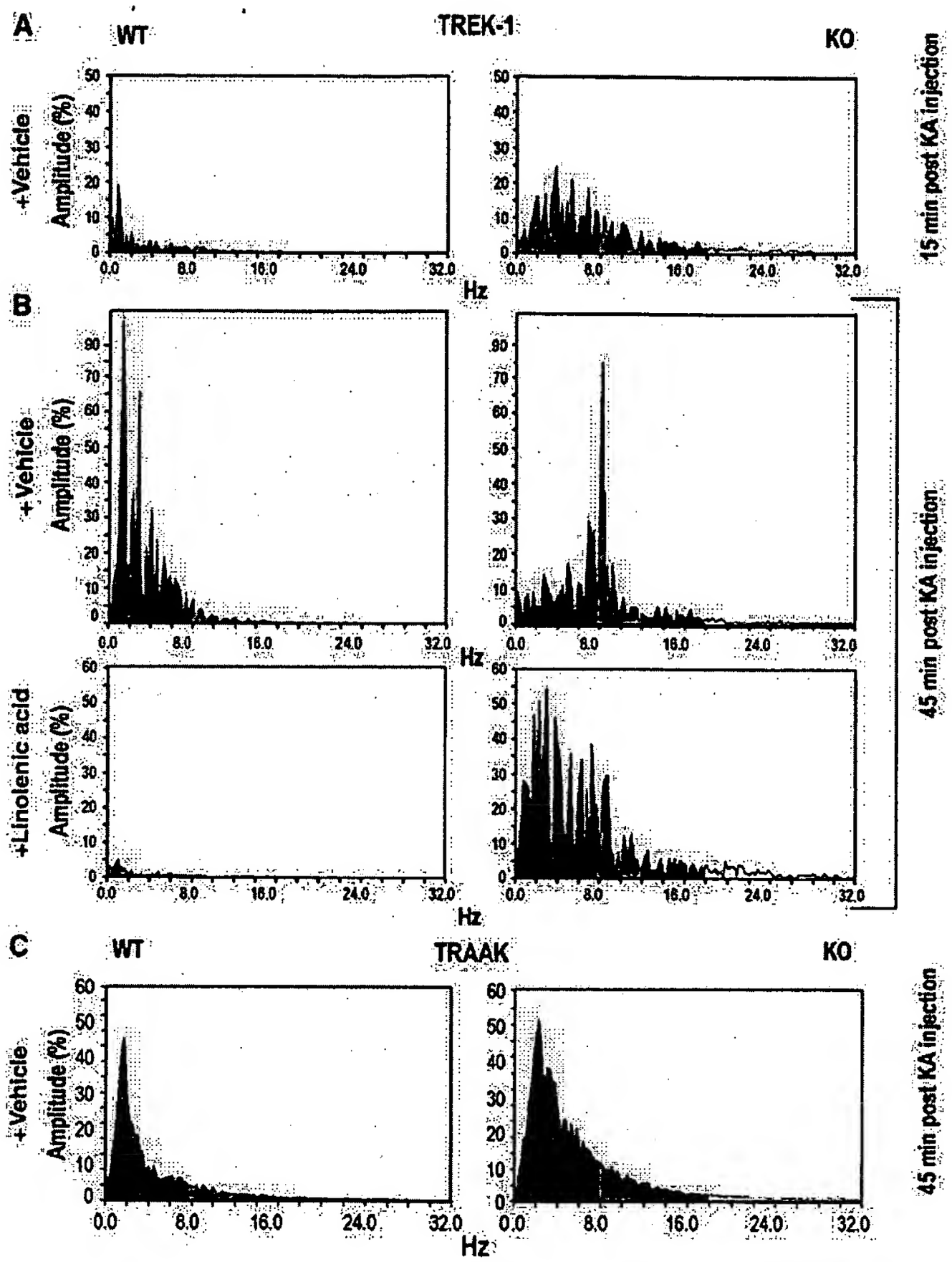

Figure 5 Spectral profiles of EEG recordings following KA (22 mg/kg) injection in Trek and Traak mice. (**A**) Increased KA susceptibility of Trek1$^{-/-}$ mice. (**B**) No anticonvulsive effect of LIN injection in Trek1$^{-/-}$ mice. (**C**) No difference in KA susceptibility between vehicle-treated Traak$^{-/-}$ (KO) and Traak$^{+/+}$ (WT) mice. Spectral profiles of EEG recordings ($n = 10$ per genotype and treatment) are shown 15 and 45 min following KA injection in vehicle-treated Trek1$^{+/+}$ and Trek1$^{-/-}$ mice and 45 min following KA injection in LIN (500 nmol/kg)-treated Trek1$^{+/+}$ and Trek1$^{-/-}$ mice. Spectral profiles of EEG recordings in vehicle-treated Traak mice are shown 45 min following KA injection.

patterns in the hippocampus of Trek1$^{+/+}$ and Trek1$^{-/-}$ mice treated with LIN (Figure 6B) and their spectral analysis of EEG activity (Figure 5B) confirm the lack of efficiency of LIN treatment in null mutant mice. The same protocol applied to TRAAK mice showed no difference in the neuroprotective effect of LIN or LPC between Traak$^{+/+}$ and Traak$^{-/-}$ mice (data not shown). This strongly suggests that the antiepileptic effect of PUFAs or LPLs is directly related to the activation of the TREK-1 channel.

Another important cause of neuronal damage is ischemia. Trek1$^{+/+}$ and Trek1$^{-/-}$ mice were submitted to a transient bilateral occlusion of common carotid arteries (CCAs) during systemic hypotension (mean arterial blood pressure (MABP) 30 ± 3 mmHg) maintained for 30 min. Trek1$^{+/+}$ mice presented no sign of hyperexcitability in the days following a 30 min period of ischemia. In contrast, most of the knockout mice developed seizures of progressive severity during the same time of reperfusion. More than 70% of Trek1$^{-/-}$ mice died in the 3 days after ischemia compared with 34% of Trek1$^{+/+}$ mice (Figure 6C; $P<0.001$). LIN or LPC (500 nmol/kg) injected 30 min before the induction of global ischemia had no effect in Trek1$^{-/-}$ mice, while it protected the Trek1$^{+/+}$ mice against neuronal death and significantly increased their survival (Figure 6C). This observation strongly suggests that the neuroprotective effect of PUFAs or LPLs against global ischemia is directly related to the activation of the TREK-1 channel. The specificity of the TREK-1 channel in neuroprotection against ischemic injury is strengthened by results obtained with TRAAK-deficient mice, which did not display an increased sensitivity to ischemia (Figure 6C).

We also analyzed the role of TREK-1 in spinal cord ischemia. It is a devastating complication with resulting paraplegia, observed after repair of thoracic or abdominal

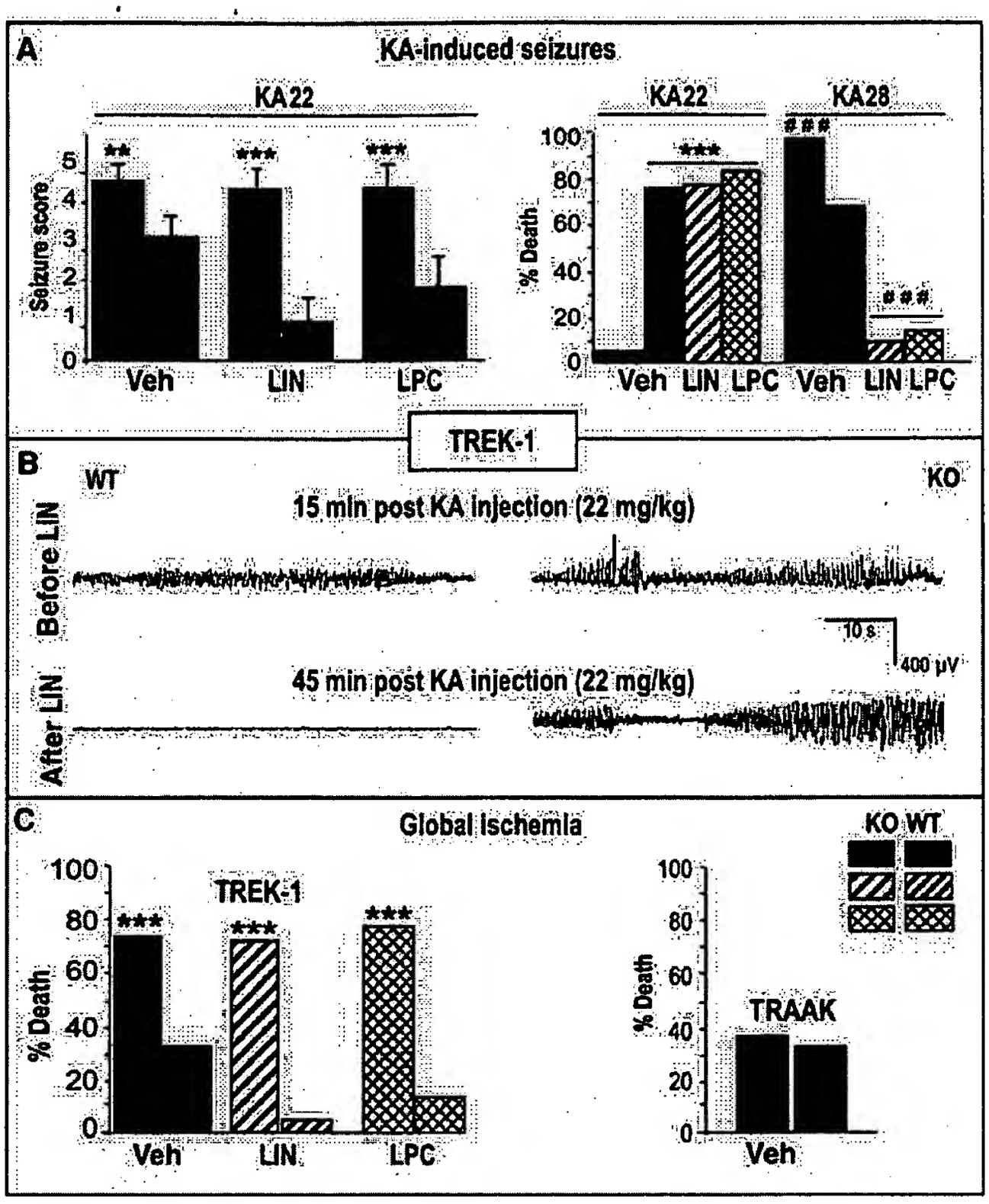

Figure 6 Increased vulnerability of TREK-1-deficient mice to ischemia and loss of the neuroprotective effect of LIN and LPC in Trek1$^{-/-}$ mice. (**A**) Effect of LIN or LPC injection (500 nmol/kg) 10 min before KA treatment. (**B**) EEG recordings (15 and 45 min after KA treatment) in Trek1$^{+/+}$ and Trek1$^{-/-}$ mice with or without LIN (500 nmol/kg). (**C**) Increased mortality rate in vehicle (Veh)-, LIN- or LPC-treated Trek1$^{-/-}$ mice following 30 min global ischemia ($n = 20$ per genotype). LIN and LPC were injected at a concentration of 500 nmol/kg 30 min before ischemia. *Significantly different from vehicle-treated wild type (KA treatment 22 mg/kg), #significantly different from vehicle-treated wild type (KA treatment 28 mg/kg), **$P<0.001$, ***$P<0.0001$, ###$P<0.0001$, ANOVA followed by Tukey's multiple comparison test.

aortic aneurysms or dissection (Kouchoukos and Dougenis, 1997). Combined occlusion of the aortic arch and left subclavian artery was performed to induce spinal cord ischemia in mice (Lang-Lazdunski *et al*, 2000). Values of mean femoral arterial blood pressure (MABP) recorded for 5 h throughout the procedure did not differ significantly between Trek1$^{+/+}$ and Trek1$^{-/-}$ mice (Table Ia). The susceptibility to spinal cord ischemia was much higher in null allele mice. A total of 75% of Trek1$^{-/-}$ mice died within the first 3 h following 10 min ischemia compared with 14% of Trek1$^{+/+}$ mice up to 24 h after the procedure (Table Ib; $P<0.001$). All surviving Trek1$^{+/+}$ mice recovered without any neurological deficit and failed to develop any form of neurological deficit during the subsequent 48 h. In contrast, surviving Trek1$^{-/-}$ mice developed severe hind limb paralysis at the onset of reperfusion. They remained paralyzed during the first hours of reperfusion and retained deficits in motor function during the subsequent 48 h (Table Ib). Within 5 min following aortic crossclamping, Trek1$^{-/-}$ mice had vesical relaxation with urination, which did not occur in Trek1$^{+/+}$ mice, further indicating a lower tolerance to spinal cord ischemia. Autopsies of Trek1$^{-/-}$ mice did not reveal any severe abnormality in heart, lungs or major vessels.

TREK-1 channel in the mechanism of action of volatile anesthetics in vivo

Another interesting property of the TREK-1 channel concerns its sensitivity to activation by general volatile anesthetics (Patel *et al*, 1999), and we hypothesized (Patel *et al*, 1999) that TREK-1 might be involved in the mechanism of action of these agents. The comparative sensitivity to different volatile anesthetics of Trek1$^{+/+}$ and Trek1$^{-/-}$ mice was assessed by comparing the onset of anesthetic action, the loss of righting reflex (LORR) and the inspired minimum alveolar anesthetic concentration (MAC) values for each anesthetic in both. MAC is the minimum steady-state alveolar concentration of an inhalational anesthetic required to suppress a strong motor reaction to the noxious stimulus of tail-clamping in 50% of mice (Quasha *et al*, 1980). Figure 7A shows that knockout mice had a decreased sensitivity to chloroform and halothane, which are the most potent activators of the TREK-1 channel *in vitro* (Patel *et al*, 1999). Interestingly, the same

Table I Comparison of susceptibility to spinal cord ischemia in wild-type and TREK-1-deficient mice

(a) Physiological variables

Genotype	Mean arterial blood pressure (mmHg)			Rectal temperature (°C)		
	Preischemia	Ischemia	Reperfusion	Preischemia	Ischemia	Reperfusion
$Trek1^{+/+}$	71.9±3.3	16.1±6.2	67.4±4.2	37.5±0.4	37.3±0.5	37.6±0.3
$Trek1^{-/-}$	73.2±2.9	17.2±2.4	69.7±3.8	37.3±0.3	37.2±0.3	37.4±0.2

(b) Number of mice with their neurologic status (MSDI) and death rate at the onset of reperfusion and 1, 3 and 24 h after ischemia

Time after ischemia (h)	Genotype	MSDI							
		0	1	2	3	4	5	6	Death
0	$Trek1^{+/+}$	0	7	0	0	0	0	0	0
	$Trek1^{-/-}$*	0	0	0	0	0	0	8	0
1	$Trek1^{+/+}$	1	6	0	0	0	0	0	0
	$Trek1^{-/-}$*	0	0	0	0	0	1	3	4
3	$Trek1^{+/+}$	7	0	0	0	0	0	0	0
	$Trek1^{-/-}$*	0	0	0	1	1	0	0	2
24	$Trek1^{+/+}$	6	0	0	0	0	0	0	1
	$Trek1^{-/-}$*	0	2	0	0	0	0	0	0

The neurologic score involved a six-point scale (0 (normal function) to 6 (severe paraplegia); Lang-Lazdunski *et al*, 2000). Motor sensory deficit indices (MSDIs) were analyzed with Kruskal–Wallis test followed by Mann–Whitney U-test when significant. *$P<0.05$ versus wild-type mice.

type of results was obtained with sevoflurane and desflurane (Figure 7B), the most widely used agents in clinical anesthesia as well as isoflurane (Supplementary Figure 2C). The period of time necessary for the induction of anesthesia was longer, the concentrations required for LORR lower and the partial pressures of all anesthetics tested (i.e. MAC) were higher in $Trek1^{-/-}$ mice. There was no significant difference in the respiratory rate between either genotype before induction of anesthesia and at the MAC value (Table II). In contrast with volatile anesthetics, no difference was seen between $Trek1^{+/+}$ and $Trek1^{-/-}$ mice upon injection of the barbiturate pentobarbital (Figure 7C), which produces anesthesia by acting on different $GABA_A$ receptor subunits (Yamakura *et al*, 2001) and *in vitro* it has no effect on TREK-1 channel activity (Figure 7C), unlike halothane and sevoflurane (Supplementary Figure 2A and B). Pentobarbital did not affect the latency or the duration of LORR (Figure 7C) in null mutants. This latter result supports the idea that the differences observed are specific to volatile anesthetics and related to the TREK-1 channel.

Discussion

Potassium channels play a major role in the control of K^+ homeostasis and in physiological and pathological functions that are associated with modifications of the electrical membrane potential. Many subtypes of K^+ channels have been cloned in the past decades (Salkoff *et al*, 1992; Jan and Jan, 1997; Pongs, 1999; Kurachi *et al*, 1999). The mammalian two-pore-domain K^+ channel family (Lesage and Lazdunski, 2000; Patel and Honoré, 2001; Lesage, 2003), and particularly the TREK-1 channel, has been proposed to play a key role in brain and spinal chord injuries (Lauritzen *et al*, 2000; Blondeau *et al*, 2002; Lang-Lazdunski *et al*, 2003). The lipid and mechano-gated TREK-1 channel is closely related to pathophysiological conditions, such as ischemia and epilepsy. It is activated by arachidonic acid and other PUFAs, LPLs, cell volume expansion and internal acidosis. During the process of ischemia, arachidonic acid is released from the plasma and intracellular pH is decreased. These condition changes could potently activate the lipid-sensitive mechano-gated K_{2P} channels, an activation that would occur to protect the neuronal cell against excessive and deleterious neuronal excitability and Ca^{2+} entry. On the other hand, the TREK-1 channel is inhibited by the activation of group I metabotropic glutamate receptors, known to be involved in brain disorders, including ischemia, epilepsy and neurodegenerative disorders (Bockaert *et al*, 1993; Bordi and Ugolini, 1999; Fagni *et al*, 2000). Group I metabotropic glutamate receptor antagonists are neuroprotectors, while agonists amplify the excitotoxic neuronal degeneration induced by glutamate (Nicoletti *et al*, 1996; Gasparini *et al*, 2002). In fact, injections of PUFAs and LPLs protect against brain and spinal chord ischemia as well as epileptic seizures (Lauritzen *et al*, 2000; Blondeau *et al*, 2001, 2002; Lang-Lazdunski *et al*, 2003). Riluzole, another activator of TREK-1 channel (Duprat *et al*, 2000), is also neuroprotective against ischemia (Pratt *et al*, 1992; Ettaiche *et al*, 1999; Lang-Lazdunski *et al*, 1999). Although the opening of lipid-sensitive mechano-gated K_{2P} channels has been presumed to be the significant factor in neuroprotection, the lack of specific blockers did not allow until now a direct demonstration of this property. Using mice with disrupted TREK-1 and TRAAK genes, the present study provides evidence for a major role of the TREK-1 channel in surviving excessive neuronal excitability and in resistance to forebrain and spinal cord ischemia. The absence of an increased sensitivity to ischemia and epilepsy in $Traak^{-/-}$ mice demonstrates that the extreme vulnerability of $Trek1^{-/-}$ mice is not a nonspecific effect due to the lack of an important K^+ channel on neuronal excitability. Consequently, the TREK-1 channel can be considered to play a key role in the regulation of neuronal excitability. The high expression of the TREK-1

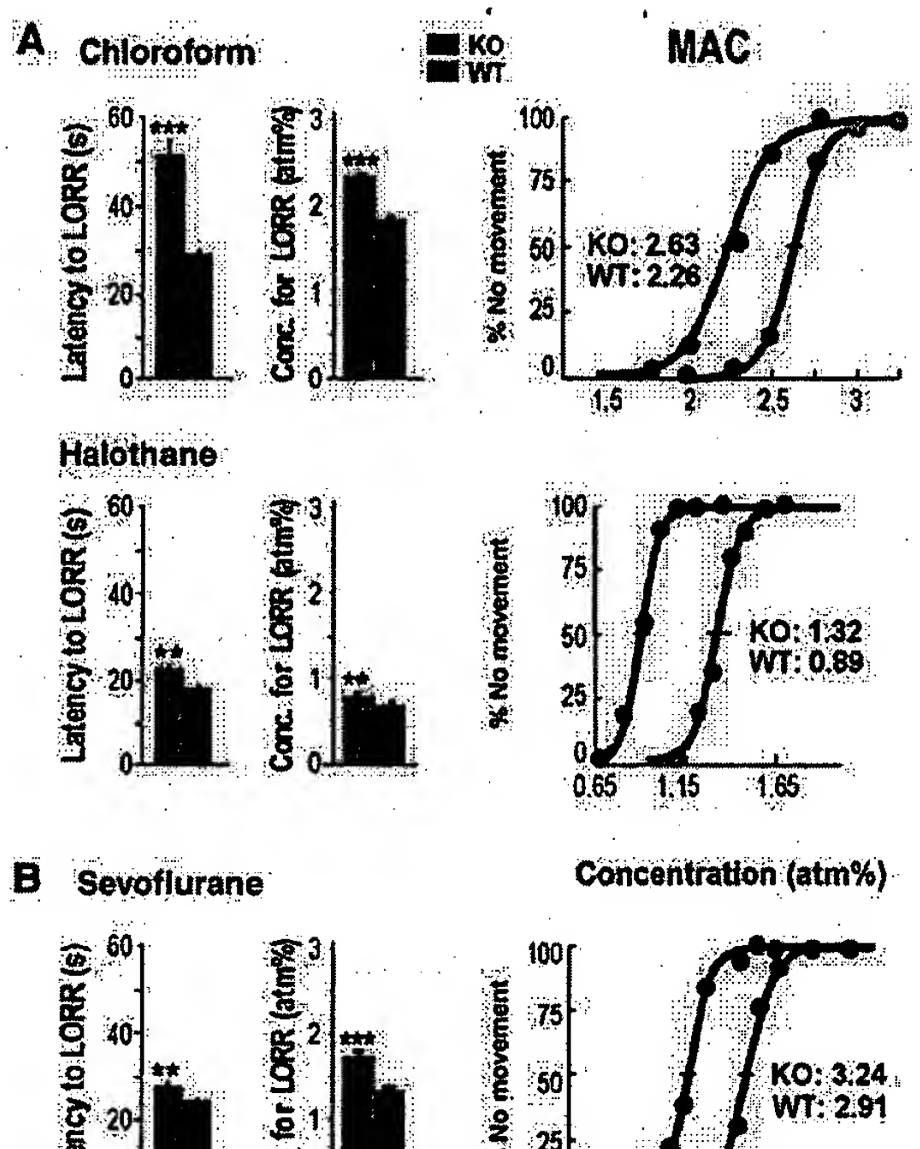

Figure 7 Effects of different anesthetics on LORR and MAC in Trek1$^{+/+}$ and Trek1$^{-/-}$ mice. LORR measurements after inhalation of volatile anesthetics. Latency to LORR is defined as the period of time (s) from inhalation to the LORR. Concentration for LORR corresponds to average concentrations of volatile anesthetics (**A**) chloroform and halothane and (**B**) sevoflurane and desflurane for the recovery from LORR. (**C**) LORR measurements (latency and duration of LORR expressed in minutes) after pentobarbital injection (30 mg/kg). Lack of effect of pentobarbital (2.4 mM) on TREK-1 channel expressed in transfected COS cells. *I–V* curves in steady-state control condition and after a 5 min application of pentobarbital (2.4 mM). *I–V* curve was elicited by a voltage ramp (1 s duration from −130 to +100 mV). Data represent mean±s.e.m. ($n=20$ per genotype and anesthetic agent). Statistical significance (Student's *t*-test): $^{**}P<0.001$, $^{***}P<0.0001$. Logistic regression probability of no movement fitted for volatile anesthetic concentrations. MAC and its 95% confidence interval (horizontal line) are shown on each graph.

protein both pre- and postsynaptically in the cortex and thalamic nuclei is consistent with a potential role for this channel in prevention of epileptic seizures. The high levels of TREK-1 expression in the hippocampus, a structure susceptible to damage during ischemia, and its modulation by neurotransmitter receptor activation are supplementary arguments for a major role of this channel in the control of excitotoxicity. Its activation in the neurons would be expected to hyperpolarize synaptic terminals, decreasing glutamate release and/or producing a postsynaptic hyperpolarization, which would favor the blockade of the NMDA receptor-associated channel by Mg^{2+} and also counterbalance glutamate-induced depolarization on other types of ionotropic glutamate receptors (Lauritzen *et al*, 2000). Without excluding a localization of the TREK-1 protein in glutamatergic neurons (Lauritzen *et al*, 2000), the TREK-1 channel has been described to be colocalized in GABAergic interneurons, specifically from striatum (this work), cerebellum, cortex and hippocampus (Hervieu *et al*, 2001). The phenotype of extreme vulnerability of TREK-1 null mutants against epilepsy and ischemia is consistent with the absence of TREK-1 channel in GABAergic interneurons, known to serve inhibitory functions in CNS and be involved in ischemic and epileptic disorders (Treiman, 2001; Wang, 2003). In the light of the role of TREK-1 channels in setting resting membrane potential, this is suggestive that TREK-1 may set the membrane potential of interneurons and thereby contribute to their often distinctive neurophysiological properties.

The beneficial effects of PUFAs on human health have long been advocated (Leaf and Kang, 1996; Nair *et al*, 1997; Leaf *et al*, 1999; Nordoy, 1999; Stoll *et al*, 1999) and indeed the effects of PUFAs on neuroprotection against epilepsy and ischemic paradigms in animals are spectacular (Lauritzen *et al*, 2000; Lang-Lazdunski *et al*, 2003). The results presented here strengthen the idea that neuroprotection induced by PUFAs (and LPLs) against seizures and ischemia is related to their action on the TREK-1 channel since this neuroprotection disappears in Trek1$^{-/-}$ mice and open the way for a novel neuroprotective strategy.

The possibility that a significant part of the effects of general anesthetics might result from potassium channel activation and especially K_{2P} channels has been previously suggested (Patel *et al*, 1999). This work definitively shows that the deletion of the TREK-1 gene induces a resistance to volatile anesthetics. This resistance is actually the greatest found for any ion channel knockout tested, including knockouts of $GABA_A$ receptors (Campagna *et al*, 2003), also believed to be potential targets of volatile anesthetics. One might of course wonder why the deletion of TREK-1 does not completely abolish sensitivity to volatile anesthetics. An important reason is that volatile anesthetics such as halothane, desflurane and sevoflurane also activate other K_{2P} channels such as TREK-2 and TASK channels (Patel *et al*, 1999; Lesage *et al*, 2000), which are still expressed in the Trek1$^{-/-}$ mice. It will be important in the future to analyze multiple K_{2P} channel knockouts, which would then be expected to display extreme resistance to volatile anesthetics. Further experiments using selective Cre mice to abolish specifically the gene in a tissue or a cell type will also permit a more detailed analysis of the cellular mechanisms that underlie the behavioral responses.

Table II Respiratory rate (beats/min) of wild-type and TREK-1-deficient mice before the induction of anesthesia and at the MAC value

	Chloroform	Halothane	Isoflurane	Sevoflurane	Desflurane
Wild-type preanesthesia	152±4	160±1	159±2	155±3	163±1
Wild-type MAC	166±4	141±2	84±4	105±3	106±3
Knockout preanesthesia	154±2	156±2	161±2	152±3	160±2
Knockout MAC	174±3	148±4	96±5	116±3	111±3

Data were expressed as mean±s.e.m. Statistical significance between wild-type and knockout mice was set at $P<0.05$.

In conclusion, this work provides evidence for a major involvement of the TREK-1 channel in the control of the neuronal excitability and neuroprotective effects induced by PUFAs and LPLs against ischemia and epileptic seizures. TREK-1 appears to be an innovative target for the development of novel therapeutic neuroprotective strategies for brain pathologies.

Materials and methods

All experiments were conducted according to the policies on the care and use of laboratory animals of the Society of Neurosciences.

Generation of TREK-1-deficient mice

Trek-1 genomic clones were isolated from a 129 mouse genomic library by using a TREK-1 cDNA probe and subcloned into pBluescript SK (Stratagene). The floxed targeting vector was generated from a 7.5 kb *BglII*/*Eco*RI restriction fragment containing exons 1–3 of the KCNK2 gene. The vector was designed to allow CRE-mediated deletion of exon 3, which encodes the TM1 domain of the channel. The first loxp sequence was inserted in the 5′ flanking intron of exon 3. Similarly, the PGK-neomycin resistance cassette (neo) was inserted together with a second loxp sequence in the 3′ flanking intron of exon 3. Both loxp sequences were in the same orientation to allow CRE-mediated simultaneous excision of Exon 3 and neo cassette. A copy of the diphteric toxin gene was subcloned adjacent to the homologous region for negative selection of the ES clone. The targeting vector (50 μg) was linearized prior to electroporation into 129-derived embryonic stem cells. After drug selection (G-418, 350 μg/ml), one positive clone (1/288) was identified by Southern blot and PCR analysis. Five highly chimeric males were generated by injection of the targeted ES cells into C57Bl/6J blastocysts. They were mated with C57Bl/6J females and germline transmission was assessed by Southern blot and PCR analysis of tail DNA from the agouti pups. TREK-1 floxed mice were then crossed with mice carrying the CRE recombinase gene under the control of the ubiquitous CMV promoter (D Metzger). Heterozygous TREK-1-deficient mice were then backcrossed with C57Bl/6J congenic mice over 11 generations. All animals (+/+ and −/−) were 8- to 10-week-old males of N6F2 to N11F2 backcross generation.

Kainate and pentylenetetrazol administration

After intraperitoneal injection of KA at 22 or 28 mg/kg, mice ($n=20$ per group) were monitored for 2 h for onset and extent of seizures. Seizure severity was blindly scored (Tsirka *et al*, 1995). PTZ was injected similarly at 40 or 55 mg/kg and seizures were scored based on the highest degree of seizure within 15 min of the PTZ injection. The seizure index was calculated by averaging the points for seizure activity in each group ($n=20$ per genotype and treatment). EEGs were recorded for 2 h on conscious mice ($n=10$ per genotype and treatment) using four small platinum electrodes (diameter 0.28 mm) placed in the hippocampus (1.2 mm lateral, 1.6 mm posterior to the bregma, 1.6 mm inside) and in the anterior neocortex (2 mm lateral, 0.5 mm anterior to the bregma, 1.5 mm inside). The signals were amplified, digitized and quantified using the Galileo system (Sirius BB, Medical Equipment International).

Forebrain ischemia model (2 VO+hypotension)

Global ischemia ($n=20$ per genotype and treatment) was induced by occluding both CCAs with aneurysm clips (Aesculap, Germany) during a 30 min episode of systemic hypotension induced by withdrawal of blood to maintain an MABP of 30±3 mmHg (Sheng *et al*, 1999).

Spinal cord ischemia model

Mice were subjected to crossclamping of the aortic arch, left subclavian artery and internal mammary artery for 10 min (Lang-Lazdunski *et al*, 2000). Motor function was blindly evaluated in the hind limbs using a rating scale of 0 (normal function) to 6 (total absence of movement) (Lang-Lazdunski *et al*, 2000).

Behavioral studies of sensitivity to anesthetic agents

Loss of righting reflex. Unrestrained mice ($n=10$ per genotype and volatile anesthetic) were placed in a chamber maintained at 33–35°C. Carbon dioxide pressure (<0.05 atm) and rectal temperature (36.5±1.2°C) were controlled. Each volatile anesthetic (chloroform, halothane, isoflurane, sevoflurane and desflurane) was administered with a calibrated vaporizer in 100% oxygen as the carrier gas with a fresh gas flow of 2 l/min at initial concentrations of 3.0, 1.2, 1.0, 1.8 and 5%, respectively. Concentrations of the volatile anesthetic were continuously measured by using a calibrated infrared analyzer (RGM 5250, Ohmeda, Louisville). After equilibration for 20 min at each initial anesthetic concentration, mice were blindly scored for LORR. The concentration of the anesthetics was then decreased in 10–20% increments and allowed to re-equilibrate at each concentration. Mice were observed continuously for recovery of the righting reflex. The concentration reported for LORR was calculated by averaging the two concentrations at which the mouse either retained or lost the righting reflex. Data were reported as mean±s.e.m. Differences were evaluated using an unpaired *t*-test.

Tail-clamp/withdrawal assay. MAC was determined using the tail-clamp technique (Quasha *et al*, 1980). Mice ($n=20$ per genotype and volatile agent) were first exposed for 20 min to a constant anesthetic concentration of almost 50% anesthetic induction values used in clinical practice. A hemostatic clamp was applied for 45 s to the midportion of the tail. Mice were scored blind for a motor withdrawal in response to clamping the tail. A mouse was considered to have moved if it made a purposeful muscular movement of the hind limb and/or the body. The anesthetic concentration was decreased in steps of 0.1% for each anesthetic, and the testing sequence was repeated after 20 min of exposure to each concentration. Concentration–response data were fitted to a logistic equation, yielding half-effect concentrations (median MAC values), slopes and estimates of their respective standard errors. Median MAC values were given with their respective 95% confidence interval limits. All *P*-values were two-tailed, and a *P*-value <0.05 was considered significant.

Sleep time assay (i.e. duration of the LORR). Mice ($n=20$ per genotype and anesthetic agent) were blindly tested for the duration of LORR (i.e. sleep time) in response to an intraperitoneal injection of pentobarbital (30 mg/kg). Mean sleep times for each agent were compared in null allele and wild-type mice using an unpaired *t*-test.

Onset of volatile and intravenous anesthetic action (i.e. latency to the LORR). Mice ($n=10$ per genotype and anesthetic agent) were exposed to 8% chloroform, 4% halothane, 8% sevoflurane, 3% isoflurane or 10% desflurane in the same chamber used for LORR and tail-clamp assays. Onset of anesthetic action was defined as the time interval between the beginning of the anesthetic inhalation or the injection of the intravenous agent and the LORR.

Electrophysiology on COS cells
COS cells were seeded at a density of 20 000 cells per 35-mm dish 24 h before transfection. Cells were transiently transfected by the classical DEAE–dextran method with 0.1 μg pCI-mTREK-1 +0.05 μg pCI-CD8. Transfected cells were visualized 48 h after transfection using anti-CD8 beads. The external solution contained (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$ and 10 HEPES, adjusted to pH 7.4 with NaOH. The pipette solution contained (in mM) 140 KCl, 4 $MgCl_2$, 5 EGTA and 10 HEPES (pH 7.2). The cell under study was continuously superfused with a microperfusion system (0.1 ml/min) at room temperature.

Electrophysiology on mouse striatal neurons
Primary culture of mouse striata was carried out according to Weiss *et al* (1986). Cells were plated in culture dishes previously coated with polyornithin and 50% fetal calf serum. Culture medium was DMEM plus glucose (1.5 g/l) for the first 24 h, then B27 plus uridine (2 μM) and 5-fluoro-2′-deoxyuridine (2 μM). Patch clamp measurements were performed 2 or 3 days after plating. In outside-out configuration, the internal solution contained (in mM) 155 KCl, 3 $MgCl_2$, 5 EGTA, 10 HEPES and 5 ATP-K^+ (pH 7.2) and the external solution contained (in mM) 120 NaCl, 5 KCl, 3 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES. We daily prepared and added to the external solutions 10 mM tetra-ethyl-ammonium chloride, 3 mM 4-aminopyridine, 10 μM glibenclamide and 5 mM glucose (pH at 7.4). TREK current anesthetic sensitivity was assessed in striatal neurons and in TREK-1-expressing COS cells (Patel *et al*, 1999).

DNA extraction
Tail biopsy was lysed with proteinase K (200 μg/ml) for 5–12 h at 56°C in buffer containing 100 mM Tris (pH 8.5), 200 mM NaCl, 5 mM EDTA and 0.2% SDS. Proteinase K was heat inactivated at 95°C for 5–10 min and the lysate was then either diluted in water for PCR amplification or centrifuged to get rid of undigested material prior to ethanol precipitation for subsequent digestion by restriction enzymes.

Southern blot
For Southern blotting, genomic DNA was digested overnight with the appropriate restriction enzyme, precipitated, size fractionated on a 0.6% agarose gel and transferred onto a nylon membrane in 0.4 M NaOH. ^{32}P-labelled probe hybridization was carried out overnight at 65°C in 0.5 M Na_2Pi/5% SDS, pH 6.8.

PCR analysis
PCR reactions were performed on 1 μl of a 20–30 times water dilution of the crude tail lysate in 15 μl final volume containing 67 mM Tris–HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1.5 mM $MgCl_2$, 200 mM dNTP and 0.2 μl *Taq* polymerase (Eurobio). Conditions were as follows: for TREK-1, 94°C/3 min≫(94°C/20 s≫58°C/20 s≫72°C/35 s) × 33, oligos (see Figure 1) #1 (5′GGT GCC AGG TAT GAA TAG AG3′), #2 (5′TTC TGA GCA GCA GAC TTG G3′), #3 (5′GTG TGA CTG GGA ATA AGA GG3′); for TRAAK, 94°C/3 min≫(94°C/30 s>63.5°C/25 s>72°C/35 s) × 35, primers #1 (5′CCCTGCTCCTTCTTCCC3′), #2 (3′ATTCTTCCTTCCTCCCTTCC5′), #3 (5′TGGACGAAGAGCATCAGGG3′), #4 (5′GAGGAGCAGCCAACTT TAGC3′) (see Supplementary Figure 1).

In situ hybridization
Perfused brain sections were hybridized with specific oligonucleotide 3′-end-labelled probes (nucleotides 726–694 and 1536–1504 of the cloned mouse TREK-1; GenBank accesssion number U73488.2).

Immunohistochemistry
Immunostainings were performed on floating brain sections (50 μm) using the anti-rabbit α-TREK-1 (Lauritzen *et al*, 2000) and c-fos (Oncogene) rabbit polyclonal antibodies. Sections were floated in a solution of the primary antibody overnight at 4°C (1:200 dilution). Biotinylated secondary antibodies were amplified using a rabbit IgG Vector Elite ABC kit (Vector laboratories) with 3-diaminobenzidine as substrate.

TaqMan assays (real-time quantitative RT–PCR analysis)
Total RNA from the brain and cerebellum of Trek1$^{-/-}$ and Trek1$^{+/+}$ mice was isolated by using the Trizol method (InVitrogen). Reverse transcription was performed with 2 μg of total RNAs, treated for 30 min with RQ1 DNase I (Promega) and reverse-transcribed with Superscript II reverse transcriptase (InVitrogen). Real-time PCR analysis (SYBR Green Mastermix Plus, Eurogentec) was performed to estimate the level of expression of TREK-1, TREK-2, TRAAK, TASK-1, TASK3, TWIK-1 and GABAα6 subunit in the brain and cerebellum of Trek1$^{-/-}$ and Trek1$^{+/+}$ mice. Primers for the seven different amplicons were as follows:

TREK-1 forward TTTTCCTGGTGGTCGTCCTC;
TREK-1 reverse GCTGCTCCAATGCCTTGAAC;
TREK-2 forward CCGGAATTACTCTCTGGATGAAGA;
TREK-2 reverse CATGGCTGTGCTGGAGTTGT;
TRAAK forward CCCCAGTGAGAATCTGGCC;
TRAAK reverse GGGCACAGCCACGCTC;
TASK-1 forward CGGCTTCCGCAACGTCTAT;
TASK-1 reverse TTGTACCAGAGGCACGAGCA;
TASK-3 forward GACGCCCTCGAGTCGGACCA;
TASK-3 reverse CTCTGAGACGGACTTCTTC;
TWIK-1 forward TGTCCTTCTCCTCCGTCACTG;
TWIK-1 reverse AGGCCACAAAAGGCTCACTTT;
GABAα6 forward CGCCCCCTGTGGCAA;
GABAα6 reverse TACTTGGAGTCAGAATGCACAACA;
CYCLOPHILIN forward GGCTCTTGAAATGGACCCTTC;
CYCLOPHILIN reverse CAGCCAATGCTTGATCATATTCTT.

Real-time PCR assays for each gene target were performed on cDNA samples in 96-well plates on an ABI Prism 7700 Sequence Detection System (PE Biosystems). PCR data were captured using Sequence Detector Software. Data were analyzed using the comparative CT method where the amount of target was normalized to an endogeneous reference (cyclophilin D) and calibrated to the amount of target in wild-type mice (User Bulletin No. 2 Applied Biosystems). Experiments were performed in triplicate. Standard curves were generated for each set of primers using serial dilutions of mouse brain cDNA to ensure a high efficiency of amplification.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online.

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SENSITIVITY OF K+ CHANNEL TASK-1 DEFICIENT MICE TO ANESTHETICS ISOFLURANE AND PROPOFOL

A.M.Linden1*; I.M.Aller2; O.Vekovischeva1; E.Leppä1; P.H.Rosenberg3; W.Wisden2; E.R.Korpi1

1. Inst. Biomedicine, Univ. Helsinki, Helsinki, Finland

2. Dept. Clin. Neurobiology, Univ. Heidelberg, Heidelberg, Germany

3. Dept Anaesthesiology and Intensive Care Med., Helsinki Univ. Hosp., Helsinki, Finland

TASK-1 two-pore-domain K+ channel mediates background K+ currents and regulate the resting membrane potential in neurons. It is activated by inhalation anesthetics and inhibited by cannabinoid agonists. TASK-1 knockout mice were generated and backcrossed to C57BL/6 mouse line. Here, we performed SHIRPA primary behavioral characterization and tested the sensitivity of TASK-1 knockout mice for general anesthetics (isoflurane, propofol), ethanol and cannabinoid agonist WIN55212-2. In SHIRPA primary screen, the knockout mice showed reduced ($p<0.01$) startle response to sound and withdrawal response to toe-pinch compared to wild-type littermates. Inhalation anesthetic isoflurane was slightly less effective ($p<0.05$) in inducing loss of righting reflex in knockouts. However, the concentration at which wild-type and knockout mice lost tail clamp withdrawal reflex was identical. TASK-1 knockout males, but not females, were more sensitive to motor impairing effects of another anesthetic propofol in rotarod test. Knockout mice were also more ($p<0.05$) sensitive to anesthetic effects of propofol (200 mg/kg, i.p.) measured as a duration of loss of righting reflex. In contrast, TASK-1 knockouts were significantly less sensitive to the sedative effects of ethanol (3 g/kg, p.o.) and WIN 55212-2 (8 mg/kg, s.c.) in staircase exploration test. These results indicate that TASK-1 knockout mice were grossly normal although they may have minor deficits in sensory functions. Our results also suggest that TASK-1 plays only a minor, if any, role in the anesthetic effects of isoflurane. Alterations in sensitivity to propofol, ethanol and WIN55212-2 observed here warrant further pharmacological characterization of TASK-1 knockout mice.

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Headnotes

PATENTS

[1] Patentability/Validity — Specification — Enablement (§115.1105)

Claims in application for transgenic carp are not unpatentable for lack of enablement, even though specification discloses success rate of only one percent for integration of desired gene into fish embryos, since some experimentation may be required, provided it is not "undue,"and there is no evidence that one skilled in art would regard this success rate as indicating undue experimentation, since record appears to reflect need for repetitive procedure, rather than undue experimentation, and since applicants' disclosure explicitly describes methodology used to obtain claimed transgenic carp; examiner's concerns relating to reproducibility of exact carp, phenotypic characteristics, levels of expression, and reproducibility of identical fish are misplaced, since claims do not include or require these characteristics.

[2] Patentability/Validity — Specification — Enablement (§115.1105)

Need to compare claimed product with products of prior art is not relevant to question of whether

disclosure is enabling for claimed subject matter, and enablement provisions of 35 U.S.C. §112 therefore do not require applicants to recite, in claims for transgenic carp, any readily apparent altered or new phenotypic characteristic conferred by transgene as compared to other fish.

[3] Patentability/Validity — Obviousness — Combining references (§115.0905)

Application claim for transgenic carp is unpatentable for obviousness over combination

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of three prior art references, since primary reference describes how to use genetic material to modify carp, suggests how explicitly disclosed process could be modified using fish genes, and provides sufficient evidence to suggest that it would be successful, and since secondary references demonstrate that gene or genetic material required to obtain claimed transgenic carp were known at time of invention.

[4] Patentability/Validity — Obviousness — Combining references (§115.0905)

Application claims for transgenic carp are not unpatentable for obviousness over prior art references that disclose existence of gene libraries for rainbow trout, since there is no evidence that claimed amino acid sequence or nucleotide sequences were known at time of invention, and since claims cannot be held obvious on ground that one of ordinary skill in art could identify or obtain genetic material of claims by researching gene libraries disclosed in references.

[5] Patentability/Validity — Obviousness — Relevant prior art — Particular inventions (§115.0903.03)

Patentability/Validity — Specification — Enablement (§115.1105)

Finding application claims for transgenic carp prima facie obvious does not require application of prohibited "obvious to try" standard, since prior art reference provides explicit description of methodology to be employed in genetically transforming fish, and provides disclosure which would enable one skilled in art to make and use claimed transgenic fish.

Case History and Disposition
Patent application of Thomas T.S. Chen, Rex A. Dunham, and Dennis A. Powers, serial no. 08/074,972. Applicants appeal from final rejection of claims in application. Affirmed in part and reversed in part.

[Editor's Note: The Board of Patent Appeals and Interferences has indicated that this opinion is not binding precedent of the board.]

Attorneys:
Paul N. Kokulis, Dante J. Picciano, and Ann S. Hobbs, of Cushman, Darby & Cushman, Washington, D.C., for applicants.

Judge:
Before William F. Smith, administrative patent judge, McKelvey, senior administrative patent judge, and Robinson, administrative patent judge.

Opinion Text

Opinion By:
Robinson, J.

DECISION ON APPEAL

[Unpublished] This is an appeal under 35 U.S.C. §134 from the final rejection of claims 1, 3-8, and 10. Claims 11-34 stand withdrawn from consideration by the examiner and are not presented on appeal.

[Unpublished] Claims 1, 3, 7, 8, and 10 are illustrative of the subject matter on appeal and read as follows:

[Unpublished] 1. A transgenic carp whose germ cells and somatic cells contain exogenous rainbow trout growth hormone (rtGH) gene operably linked to a promoter, and introduced into said fish at an embryonic stage.

[Unpublished] 3. The transgenic carp of Claim 1, wherein said rtGH gene is encoded by an isolated DNA, a recombinant nucleic acid encoding a cDNA of said rtGH gene or a recombinant nucleic acid encoding a genomic DNA of said rtGH.

[Unpublished] 7. The transgenic carp of Claim 1 or 3 wherein all of said germ cells and somatic cells contain said rtGH gene.

[Unpublished] 8. The transgenic carp of Claim 1 or 3, wherein said carp is mosaic.

[Unpublished] 10. A transgenic progeny descended from the carp of Claim 1 or 3, the cells of which retain a copy of said introduced rtGH gene.

[Unpublished] The references relied upon by the examiner are:

[Unpublished] Agellon et al. (Agellon), "Rainbow Trout Growth Hormone: Molecular Cloning of cDNA and Expression in *Escherichia coli," DNA,* Vol. 5(6), pp. 463-471 (1986).

[Unpublished] Maclean et al. (Maclean), "Introduction of Novel Genes into Fish," *Bio/Technology,* Vol. 5, pp. 257-261 (1987).

[Unpublished] Wilmut et al. (Wilmut), "A Revolution in Animal Breeding," *New Scientist,* Vol. 119 (1620), pp. 56-59 (1988).

[Unpublished] Van Brunt, "Molecular Farming: Transgenic Animals as Bioreactors," *Bio/Technology*, Vol. 6 (10), pp. 1149-1154 (1988).

[Unpublished] Gonz'alez-Villasen'or et al. (Gonz'alez), "Molecular Cloning and Sequencing of Coho Salmon Growth Hormone cDNA," *Gene*, Vol. 65, pp. 239-246 (1988).

[Unpublished] Ozato et al. (Ozato), "Transgenic Fish: Biological and Technical Problems," *Zoological Science,* Vol. 6, pp. 445-457 (1989).

Grounds of rejection

[Unpublished] Claims 1, 3-8 and 10 stand rejected under 35 U.S.C. §112, first paragraph, as being non-

enabled by the specification or, alternatively, as being drawn to a scope of subject matter which is broader than is enabled by the specification. As evidence, the examiner relies on Ozato, Wilmut, and Van Brunt.

[Unpublished] Claims 1, 3-8, and 10 stand rejected under 35 U.S.C. §103. As evidence of obviousness the examiner relies on Maclean, Gonz'alez, and Agellon.

[Unpublished] We reverse the rejections under 35 U.S.C. §112, first paragraph, and the rejection of claims 4, 5, 6, and 7 under 35 U.S.C. §103 and affirm the rejection of claims 1, 3, 8 and 10 under 35 U.S.C. §103.

Background

[Unpublished] The appellants describe the invention, at pages 2 and 3 of the specification, as being directed to transgenic carp which have a rainbow trout growth hormone (rtGH) gene integrated into their genome. Appellants state that the fish may contain the rtGH gene in all germ-line and somatic cells or, alternatively, may be mosaic, where only a portion of the germ-line and somatic cells contain the rtGH gene.

Discussion

The Claims

[Unpublished] Claim 1 is directed to a transgenic carp which contains a rtGH gene operably linked to a promoter where the exogenous gene has been introduced into the carp at an embryonic stage. Thus, the claim is narrow in being limited to a specific fish, transgenic carp, having an exogenous gene which encodes a specific growth hormone, rtGH, present in its genome and yet broad in that the claim does not require that the carp exhibit any particular observable effect or phenotypic characteristic attributable to the expression of the exogenous gene. Further, as evidenced by dependent claims 7 and 8, claim 1 is generic and encompasses transgenic carp where all of the germ cells and somatic cells contain the rtGH gene or where only a portion of the germ cells and somatic cells contain the rtGH (Specification, page 3). Claim 3 provides that the rtGH is encoded by an isolated DNA, a recombinant nucleic acid encoding a cDNA, or a recombinant nucleic acid encoding a genomic DNA of the rtGH. Claim 4 provides that the rtGH gene has a nucleotide sequence which encodes either of the two specified amino acid sequence for the rainbow trout growth hormone. Claims 5 and 6 provide that the rtGH gene has a genomic nucleotide sequence which is structurally defined. Claim 7 requires that all of the germ cells and somatic cells of the transgenic carp contain the rtGH gene. Claim 8 provides that the transgenic carp is mosaic. Claim 10 is directed to the progeny of the carp of claim 1, the cells of which retain a copy of the introduced rtGH gene.

The rejections under 35 U.S.C. §112, first paragraph

[Unpublished] We are mindful that the Patent and Trademark Office (PTO) bears the initial burden of providing reasons for doubting the objective truth of the statements made by appellants as to the scope of enablement. Only when the PTO meets this burden, does the burden shift to appellants to provide suitable evidence indicating that the specification is enabling in a manner commensurate in scope with the protection sought by the claims. *In re Marzocchi,* 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971).

[Unpublished] In support of the rejection, the examiner cites Ozato as evidencing a level of unpredictability in this art and teaching that there are three steps or factors which must be shown to exist in a true transgenic animal. (Answer, pages 6-7). Ozato, in discussing transgenic experiments in fish lists the three steps as 1) integration into the host chromosome, 2) expression, and 3) germ-line transmission of foreign genes. (Ozato, page 446, column 1, first full paragraph). Ozato, also, notes that "[n]o single experiment has yet combined all three steps in fish." (Ozato, page 446, column 2,

first sentence). The examiner notes various perceived deficiencies in appellants'disclosure and concludes that (Answer, page 8):

> **[Unpublished]** the specification does not disclose a process that is repeatable as to the levels of expression (note the issues raised in the Ozato *et al.* reference and the above discussion of the Chen declaration) to obtain carp or other fish that expresses the same transgene product that is shown to directly effect the phenotypic characteristic properties of the fish ....

[Unpublished] For their part, the appellants do not dispute the three factor test of Ozato, but argue that the present disclosure would meet this test and permit a person skilled in this art to "make and use Appellants' claimed invention by following the detailed procedures disclosed on pages 15-25 of the present specification." (Principal Brief, page 7). As urged by appellants (Principal Brief, page 7), the specification reasonably appears to describe:

> **[Unpublished]** *Integration* of the gene for rainbow trout growth hormone into the carp ... shown in Fig. 2, in Table 1 and in Table 2. *Expression* of the integrated rainbow trout growth hormone gene in red blood cells is shown on page 22, in Example 3 and in Table 2. *Transmission* of the rainbow trout growth hormone in carp is shown on page 23, in Example 4, in Table 2 and in Table 3.

[Unpublished]

[1] In responding to appellants' arguments, the examiner urges that the level of experimentation is undue and points to the success rate 1% or 20 out of 1746 attempts for the integration of the gene into the embryos described in the specification. (Answer, pages 6 and 14). However, the examiner offers no evidence which would reasonably support a conclusion that one skilled in this art would regard this rate of success for the integration of the rtGH gene as evidencing undue experimentation. We remind the examiner that some experimentation may be required as long as it is not undue. *In re Vaeck,* 947 F.2d 488, 496, 20 USPQ2d 1438, 1445(Fed. Cir. 1991). Appellants' disclosure explicitly describes the methodology to be used to arrive at the claimed transgenic carp. As the record now stands, the numbers emphasized by the examiner would reasonably appear to reflect the need for a repetitive procedure, rather than undue experimentation by those wishing to practice the invention.

[Unpublished] The specification provides explicit instructions as to how the transgenic carp can be obtained and includes evidence that would reasonably support the appellants'conclusion that the claimed transgenic carp, meeting the three criteria of Ozato, can be obtained by following the described process. The examiner's concerns relating to reproducibility of the exact carp, phenotypic characteristics, levels of expression, and reproducibility of identical fish are misplaced, because the claims do not include or require these limitations. The appellants need only provide an enabling disclosure for the claimed invention. *In re Vaeck, supra; In re Fisher,* 427 F.2d 833, 839, 166 USPQ 18, 24(CCPA 1970). Thus, there is no requirement for a disclosure for what the examiner perceives as possible characteristics of such a product. In addition, Maclean, at page 257, column 1, relied on by the examiner as evidence relating to the state of the art relative to the claimed invention adds credence to appellants'position in stating:

> **[Unpublished]** Fish lend themselves to experimental introduction of novel genes. Fertilisation (sic, Fertilization) of eggs is external and is easily carried out by artificial stripping of cock and hen fish and mixing of eggs and milt immediately or after some delay. Eggs are numerous and in many species quite large, that is more than 1 mm in diameter, rendering injection of material by micromanipulation relatively straightforward. Eggs are easily maintained after fertilisation (sic, fertilization) and in many warm water species development is very rapid (although in the rainbow trout *(Salmo gairdneri)* development in water at 10°C may take about 24 days to hatching. Work with fish thus avoids many of the difficulties of the mammalian egg, such as difficult procurement, a brief period of possible *in vitro*

culture, and the necessity for reintroduction into the reproductive tract of a receptive female. (Footnote omitted).

[Unpublished] The examiner has also rejected claims 1, 3-8, and 10 under 35 U.S.C. §112, first paragraph, urging that (Answer, page 9):

> **[Unpublished]** the disclosure is enabling only for claims limited to transgenic carp wherein the carp which have the same levels of expression of
>
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>
> the transgene exhibit the identical phenotypic properties of increased growth and growth rate and weight as compared to the nontransgenic carp of the same age and genotype but for the inserted transgene. Here again, the examiner appears to be focusing on phenotypic properties, which are not presently claimed and which he previously has urged that the disclosure does not support.

[Unpublished]

[2] The reliance on Van Brunt and Wilmut is misplaced since both relate to mammals rather than transgenic fish and as noted in the portion of Maclean cited above, "Work with fish ... avoids many of the difficulties of the mammalian egg...." To the extent that the examiner would urge that the claims are directed to an unpredictable art since the range of expression of the transgene and the effects of its expression on the animal as a whole are not predictable (Answer, pages 7-9), we note that the claims do not require a particular level of expression of the gene for rtGH or any particular effect of expression of this gene. Further, the examiner points to no authority for requiring appellants to recite "in the claims any readily apparent altered or new phenotypic characteristic(s) conferred by the transgene as compared to other fish." (Answer, page 10). The examiner's apparent view that the appellants need to compare their claimed product with products of the prior art, while possibly relevant in an inquiry under 35 U.S.C. §103, has not been demonstrated to be relevant to the question of whether the disclosure presented is enabling for the claimed subject matter. *See In re Wands,* 858 F.2d 731, 8 USPQ2d 1400, (Fed. Cir. 1988).

[Unpublished] Thus, on the record before us, we find that the examiner has not established a reasonable basis for questioning the sufficiency of the supporting disclosure when taken in combination with the relevant state of the art as it relates to the claimed invention. Therefore, the rejections under 35 U.S.C. §112, first paragraph, are reversed.

The rejection under 35 U.S.C. §103

[Unpublished] In rejecting claims 1, 3-8, and 10 under 35 U.S.C. §103, the examiner has relied upon Maclean taken with Gonz'alez and Agellon.

[Unpublished] The examiner cites Maclean as disclosing transgenic fish, specifically rainbow trout and Atlantic salmon that have been genetically modified by incorporating DNA which encodes either human growth hormone (hGH) or rat growth hormone (rGH). (Answer, paragraph bridging pages 10-11). The examiner urges that Maclean, at page 258, further suggests the use of fish genes in fish to obtained similar results and notes that fish cDNA libraries, including rainbow trout are known and that the DNA for rtGH has been isolated. (Answer, page 11). Gonz'alez and Agellon are cited to demonstrate that rtGH and the DNA which encodes the growth hormone were known as suggested by Maclean. The examiner concludes that (Answer, page 11):

> **[Unpublished]** it would have been obvious to one or ordinary skill in the art to substitute fish GH for human GH or rat GH as MacLean et al (page 258) suggest using same ... and that the DNA for rtGH has been isolated.

[Unpublished]

[3] We would characterize Maclean as describing transgenic fish which have been genetically manipulated in an effort to produce faster growing bigger fish. (Maclean, Figure 3). Maclean describes how to accomplish this goal using genes for human growth hormone (hGH) and rat growth hormone (rGH) as the transforming genetic material. Additionally, Maclean suggests the possibility of using genes from other fish and specifically notes that the gene libraries, useful for obtaining fish genes, were available. Thus, Maclean provides the reason, suggestion, or motivation 1 to genetically modify fish, including carp, and suggests the use of genetic material from other fish in place of the explicitly disclosed hGH or rGH. Here, since the references suggest producing transgenic fish, the question becomes whether Maclean provides sufficient information to enable those of ordinary skill in this art to transform carp with the genetic material which would encode the rtGH, as presently claimed. As our appellate court stated in discussing the Polisky reference in *In re O'Farrell,* 853 F.2d 894, 902, 7 USPQ2d 1673, 1680(Fed. Cir. 1988):

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[Unpublished] Polisky contained detailed enabling methodology for practicing the claimed invention, a suggestion to modify the prior art to practice the claimed invention, and evidence suggesting that it would be successful. Maclean similarly provides a description of to how to use genetic material to modify fish, including carp, suggests how the explicitly disclosed process could be modified using fish genes and provides sufficient evidence to suggest that it would be successful. As urged by the examiner, Agellon and Gonz'alez demonstrate that the gene or genetic material which would encode rtGH or cDNA were known Gonz'alez, in particular , discloses a nucleotide sequence which is taught as encoding the rtGH. (Gonz'alez, page 242). Viewing the art as a whole, there is sufficient evidence before us which would reasonably support the examiner's determination that, at the time of the invention by appellants, it would have been *prima facie* obvious within the meaning of 35 U.S.C. §103 to use the rtGH encoding DNA of Agellon and Gonz'alez to genetically transform carp in the manner described by Maclean to arrive at the transgenic carp of claim 1. As to claim 3, which requires that the rtGH is encoded by an isolated DNA, a recombinant nucleic acid encoding a cDNA of rtGH or a recombinant nucleic acid encoding a genomic DNA of rtGH, Maclean states at page 257, column 2, second full paragraph that "Genes may be cloned either as genomic or cDNA copies" and Gonz'alez discloses the cDNA which encodes rtGH at page 242. That the resulting fish may be mosaic, as required by claim 8, is taught by Maclean at page 259, column 2, paragraph no. 6. As to the progeny of the carp of claim 1, as claimed in claim 10, Maclean again expresses the view that this is capable of being realized at page 259, paragraph no. 11. Thus, as to claims 3, 8 and 10, as with claim 1, we find that the examiner has presented sufficient evidence to support a determination that the subject matter encompassed therein would have been *prima facie* obvious to those of ordinary skill in the art at the time of the invention.

Claims 4, 5, 6:

[Unpublished]

[4] Claims 4, 5, and 6 differ from claim 1 in being directed to a transgenic carp which has a nucleotide sequence which encodes one of two specified amino acid sequences of rtGH (Claim 4) and a transgenic carp wherein the rtGH gene has the nucleotide sequence of claim specified in either claims 5 or 6. In addressing the question of obviousness as to claims 4, 5, and 6, the examiner explains how one of ordinary skill in this art could obtain, isolate, or identify the nucleotide sequences which encode the specific amino acid sequences of claim 4 or the nucleotide sequences of claims 5 and 6 given the disclosure of Gonz'alez and Agellon of gene libraries for rainbow trout. (Answer, pages 11 and 36). However, the examiner has provided no evidence which would reasonably establish that, at the time of the invention by appellants, either the amino acid sequence or claim 4, the nucleotide sequence necessary to encode the specific amino acid sequence of claim 4, or the nucleotide sequence of claims 5 and 6 were known. In arguing that the claimed invention would have been obvious since one skilled in this art would need only go "fish"in the

gene libraries shown to be available at the time in order to "catch"the genetic material of the claims, the examiner is relying on a line of reasoning which has been addressed with disfavor by our appellate reviewing court in *In re Deuel*, 51 F.3d 1552, 34 USPQ2d 1210 (Fed. Cir. 1995) and *In re Bell*, 991 F.2d 781, 26 USPQ2d 1529 (Fed, Cir. 1993). Here, the examiner provides no facts or evidence which would direct those of ordinary skill in this art to the particular nucleotide sequence required by claims 4, 5, and 6. *Compare Ex parte Goldgaber,* 41 USPQ2d 1172 (Bd. Pat. App. & Int. 1995). Thus, on this record, the examiner has failed to establish a *prima facie* case of unpatentability as to the subject matter of claims 4, 5, and 6.

Claim 7:

[Unpublished] With regard to the subject matter of claim 7, the examiner has not separately addressed the limitations of this claim and offers no evidence that the references would explicitly describe or make obvious within the meaning of 35 U.S.C. §103, a transgenic carp "wherein all of said germ cells and somatic cells contain said rtGH gene." Therefore, with regard to claim 7, the examiner has failed to establish a *prima facie* case of unpatentability of the claimed subject matter.

[Unpublished] Thus, with regard to claims 1, 3, 8, and 10 a *prima facie* case of obviousness has been established and the burden of going forward shifts to the appellants. *In re Piasecki,* 745 F.2d 1468, 1472, 14, 223 USPQ 785, 788(Fed. Cir. 1984), *In re Rinehart,*531 F.2d 1048, 1052, 189 USPQ 143, 147, (CCPA 1976).

[Unpublished] Appellants, initially, argue that (Brief, page 11):

> **[Unpublished]** the Examiner is applying the prohibited obvious-to-try standard to the transgenic carp of the present invention. The Examiner is merely stating that the method of making the transgenic carp would be obvious and that there would be a reasonable expectation of success without determining that the transgenic carp would be "obvious" from the prior-art teachings.

[Unpublished] However, as stated in *In re O'Farrell,* 853 F.2d at 903, 7 USPQ2d at 1681:

> **[Unpublished]** The admonition that "obvious to try" is not the standard under §103 has been directed mainly at two kinds of error. In some cases, what would have been "obvious to try" would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful. In others, what was "obvious to try" was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it. Neither of these situations applies here. (Citations omitted). In essence, appellants argue that Maclean does not provide an enabling disclosure which would enable a person skilled in the art to make and use the claimed transgenic fish.

[Unpublished]

[5] We do not agree. Maclean provides an explicit description of the methodology to be employed in genetically transforming fish starting at page 257, column 1, second paragraph and also at page 258, column 2, second full paragraph. In our opinion, one skilled in this art would have reasonably been able to practice the process described by Maclean given the discussion of methodology and examples provided.

[Unpublished] The appellants, also, argue (Brief, page 14) that:

> **[Unpublished]** [n]one of the cited references teach how to operably link a promoter to a rainbow trout

growth hormone gene or how to introduce the gene into carp at an embryonic state.

[Unpublished] However, Maclean recognizes the importance of the presence of a promoter for the transforming gene and suggests the use of natural intron sequences, promoter sequences and some flanking sequences, as well as the splicing to another promoter. (*Id.*). Further, we note page 258, column 1, last paragraph which states:

> **[Unpublished]** Whether the genes are of piscine or other origin it may be desirable to increase the chances of good expression by splicing the coding sequence to a strong promoter from another gene.

[Unpublished] Appellants do not argue that the teaching would not be adequate to enable those of ordinary skill in this art to practice this aspect of the genetic manipulation process in fish. Similarly, Maclean explicitly suggests that (page 258, column 2, second full paragraph):

> **[Unpublished]** as far as fish are concerned, injection into the presumed nuclear area of a newly fertilized (sic, fertilized) egg is the favoured (sic, favored) approach ....

[Unpublished] On the record before us, the examiner has provided sufficient evidence to establish a *prima facie* case of obviousness within the meaning of 35 U.S.C. §103. Having weighed appellants' arguments and evidence against the evidence of unpatentability, we hold that appellants have not established that the examiner erred in concluding that the combination of Maclean, Gonz'alez and Agellon is sufficient to establish a *prima facie* case of unpatentability as to the claimed subject matter of claims 1, 3, 8, and 10 which has not been overcome either by arguments or convincing evidence. We, therefore, affirm the rejection of claims 1, 3, 8, and 10 under 35 U.S.C. §103.

CONCLUSION

[Unpublished] The examiner's rejection of claims 1, 3-8, and 10 under 35 U.S.C. §112, first paragraph, is *reversed.* The examiner's rejections of claims 4, 5, 6, and 7 under 35 U.S.C. §103 is *reversed.*The examiner's rejection of claims

1, 3, 8, and 10 under 35 U.S.C. §103 as unpatentable over Maclean, Gonz'alez and Agellon is *affirmed.*

[Unpublished] No time period for taking any subsequent action in connection with this appeal may be extended under 37 CFR §1.136(a).

AFFIRMED-IN-PART

Footnotes

1 As stated in *Pro-Mold and Tool Co. v. Great Lakes Plastics Inc.*, 75 F. 3d 1568, 1573, 37 USPQ2d 1626, 1629 (Fed. Cir. 1996 (citation omitted)):

> It is well-established that before a conclusion of obviousness may be made based on a combination of references, there must have been a reasons, suggestion, or motivation to lead an inventor to combine those references.

- End of Case -